Remarks

Claims 1-28 are pending in the application.

Claims 1-5 and 8-10 have been rejected under 35 U.S.C. §102(b) as being anticipated by Long *et al.* (*Plant Physiol.*, 1994, 105:115); and claims 1-5, 8-10, 12-17, and 21-25 are rejected under 35 U.S.C. §103(a) as obvious over the same reference.

Applicants respectfully traverse the § 102(b) and § 103(a) rejections over Long *et al.*, set forth at pages 2-5 of the latest Office Action. In support of their traversal, Applicants reassert the cited reference is not enabling. It fails to provide the ordinary artisan with any expectation of success. In fact, the cited reference provides nothing but an invitation to experiment. Although Long *et al.* states that "Plant nitrogen metabolism has been altered by transformation with a highly active assimilatory bacterial glutamate dehydrogenase gene," no details whatsoever are provided. There is no teaching of how one would identify such a gene. No DNA sequence information is provided. No source plasmid is identified. No restriction enzyme cleavage information is provided. There is no teaching regarding source organism for the gene. No transformation vector is provided. No transformation methods are suggested. There is no teaching regarding the target plant species. In fact, there is no proof that any transgenic plant was obtained, nor even any transgenic plant cells. Although the authors assert that nitrogen metabolism in some type of purportedly transgenic plant was altered, they do not tell in what way it was altered. They speculate that "increasing the activity of plant nitrogen metabolism enzymes may alter plant growth", but maybe not. They further speculate that "increased yield and protein content . . . may result," but maybe not. They state that their unidentified bacterial GDS gene "has been altered by PCR . . . to modify the coding region "yet they provide no guidance as to what alterations were made. They assert that the 5' non-coding region of the unidentified GDS gene has been altered, but they don't tell how. They assert that the 3' non-coding region has been altered, but they don't tell how. They state that "certain codons likely to inhibit expression . . . have been altered", but they don't tell how. Finally, they conclude "The effects of the various sequence substitutions [none of which are identified] on gene expression in plant cells [unidentified] compared to the unmodified gene [unidentified] will be reported." The ordinary artisan is clearly left to speculate whether any effects were observed or not. There is no teaching that the experiments in unidentified plant cells using unidentified transformation techniques (successful?)

with an unidentified transformation vector, which may or may not have contained an unidentified bacterial GDH sequence, which was purportedly modified in a number of teasingly unspecified ways, had any observable effects at all.

It is irrefutable that to be a sufficiently anticipatory prior art reference under §102, the prior art reference must be enabling. The Court of Appeals for the Federal Circuit has stated, "when there is no disclosure of any specific starting material or of any of the conditions under which a process can be carried out, undue experimentation is required; there is a failure to meet the enablement requirement that cannot be rectified by asserting that all of the disclosure related to the process is within the skill of the art." *Genentech Inc. v. Novo Nordisk A/S*, 42 USPQ2d 1001, at 1005 (Fed. Cir. 1997). Long *et al.* discloses none of their starting materials. Long *et al.* discloses none of the conditions under which their processes were performed. In view of the foregoing, it is abundantly clear that this one paragraph abstract fails to meet the requirements of an anticipatory reference and the rejection should be withdrawn.

Further, regarding obviousness, Long *et al.*, provides nothing but the suggestion to experiment to one of ordinary skill in the art. It is woefully lacking in specifics of any kind, whether experimental procedures or resulting data, which might provide the ordinary artisan with the required reasonable expectation of success. See *KSR Int'l Co. v. Telefex Inc.*, 550 U.S. ___, 127 S. Ct. 1727 (2007). Because the reference is not enabling and fails to provide any expectation of success to the ordinary skilled artisan, no *prima facie* case of obviousness has been set forth, and the rejection should be withdrawn. Reconsideration is respectfully requested.

Next, claims 1-3, 5, 8, 10, 12-14, 16, 18-22, and 26-28 have been rejected under 35 U.S.C. §103(a) as being obvious over Coruzzi *et al.* (U.S. Patent No. 6,107,547); and claims 1-5, 8-10, and 12-28 have been rejected under 35 U.S.C. §103(a) as being obvious over Coruzzi *et al.* in view of Long *et al.*, as set forth at pages 5-6 of the latest Office Action. Applicants note that the examiner has questioned the date of the grant proposals submitted as part of the last Response. True and correct copies of the grant proposals and related correspondence in unredacted form accompany this Response. The unredacted dates show that the grant proposals are from 1991. Reconsideration in view of this is respectfully requested.

Finally, Applicants gratefully acknowledge the examiner's indication that claims 6, 7, and 11 would be allowable if resubmitted in independent form.

In view of the foregoing remarks, the applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§1.16 or 1.17 as required by this paper to Deposit Account 19-0065.

The applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,

Jeff Lloyd
Patent Attorney
Registration No. 35,589
Phone No.: 352-375-8100
Fax No.: 352-372-5800
Address: Saliwanchik, Lloyd & Saliwanchik
A Professional Association
P.O. Box 142950
Gainesville, FL 32614-2950

JL/mv/abt
Attachment: Grant Proposals

United States Department of Agriculture

Cooperative State Research Service

Awards Management Division

Washington, D.C. 20250-2200

MAY 1 3 1991

Dr. Robert R. Schmidt
Dept. of Microbiology & Cell Science
University of Florida
1053 McCarty Hall
Gainesville, FL 32611

The following proposal has been received by the Awards Management Division (AMD):

TITLE: Development of Transgenic C3 Plants Requiring Less ATP for NH4 Assimilation

Proposal Number Assigned by AMD: 9103534

Date Received: 03/20/91

Any correspondence concerning this proposal should include the above proposal number assigned by AMD, and the title.

cc: Gerald L. Zachariah

Your proposal has been received by the Nitrogen Fixation/ Metabolism Program. We expect the review process to be completed mid July 1991. In the meantime, please keep the program informed of any changes in your telephone number, status of current research and/or pending grant support.

The Panel Manager for FY91 is Dr. Paul Ludden of the University of Wisconsin-Madison.

Any correspondence should be address to:

Dr. H. Jane Smith
Program Director
Nitrogen Fixation/Metabolism Program
Room 323, Aerospace Building
L'Enfant Plaza
901 D Street, SW
Washington, DC 20250-2200
(202) 401-6030
(202) 401-6488 FAX

United States Department of Agricullture

Cooperative State Research Service

Office of Grants and Program Systems

Washington, D.C. 20250-2200

National Research Initiative Competitive Grants Program

PROPOSAL NO: 9103534
PROGRAM: National Research Initiative Competitive Grants Program
PROGRAM AREA: Nitrogen Fixation & Metabolism
TITLE: Development of Transgenic C3 Plants Requiring Less ATP for NH4 Assimilation

Dr. Robert R. Schmidt
Dept. of Microbiology & Cell Science
University of Florida
1053 McCarty Hall
Gainesville, FL 32611

JUN 25 1991

Dear Dr. Schmidt:

We regret that the National Research Initiative Competitive Grants Program (NRICGP) is unable to support your proposed research project. As a result of an inadequate budget, the NRICGP is able to fund only a fraction of the meritorious proposals received. While the scientific quality of proposals and the cost of research are constantly on the rise, the budget for agricultural research has not kept up with the trend. We are continuing our efforts to make awards that are meaningful in terms of duration and funds awarded. You can imagine, therefore, that the competition is extremely keen.

In evaluating each proposal, several factors were considered, of which scientific merit was the most important. Your fellow scientists spent much time and effort in evaluating your proposal and many of them offer constructive comments in their reviews. These comments will be made available to you upon written request to:

Program Area: Nitrogen Fixation & Metabolism
National Research Initiative
Room 323 Aerospace Building
Washington, D.C. 20250-2200

Or you may FAX your request to:

Program Area: Nitrogen Fixation & Metabolism
FAX #: 202-401-6488

Upon receiving this request, reviews and panel summary will be sent to you in due course.

Sincerely,

Paul Stumpf

PAUL STUMPF
Chief Scientist

Identical letter to Dr. Gerald L. Zachariah

Panel Summary
9103534
Schmidt

The panel was impressed with excellent progress made in the previous granting period in the definition of the biochemistry and genetics of NADP-specific GDH of *Chlorella sorokiniana*. The current proposal which would transform a GOGAT-/GS- *Arabidopsis* genotype with the *Chlorella* GDH is exciting and generally well conceived, but the energetic advantage conferred appears to be slight, as discussed in the reviews. While the substitution of Chlorella GDH for GS/GOGAT addresses the kinetic problem of higher plant GDH, i.e, the high Km for ammonia, it does not address the thermodynamic problem, i.e., the Keq for the GDH reaction. The GDH reaction will proceed toward release of ammonia under most physiological conditions. The panel also noted a critical problem in the proposal relating to the survivability of transformed GOGAT-/GS- *Arabidopsis*.

THE UNIVERSITY OF FLORIDA
INSTITUTE OF FOOD AND AGRICULTURAL SCIENCES
DEPARTMENT OF MICROBIOLOGY AND CELL SCIENCE
3103 McCARTY HALL / 100 IFAS / GAINESVILLE, FLORIDA 32611-0100 / TEL. 904/392-1906

July 1, 1991

Program Area: Nitrogen Fixation and Metabolism
National Research Institute
Room 323 Aerospace Building
Washington DC 20250-2200

Dr. Dr. Stumpf:

Thank you for your letter of June 25, informing me that my grant proposal (No. 9103534), "Development of Transgenic C_3 Plants Requiring Less ATP for NH_4^+ Assimilation", was not approved for funding.

Please send me the reviewers' comments so that I can use them to revise my proposal and resubmit it for the next grant deadline.

I am naturally very disappointed to have my proposal denied funding. This is the first grant proposal that I have had "turned down" since the initiation of the Competitive Grants Program. I believe the research described in this proposal is of importance both to basic research and to agricultural plant biotechnology.

Sincerely yours,

Bob Schmidt

Robert R. Schmidt
Graduate Research Professor

RRS/ms

UNIVERSITY OF FLORIDA

SPONSORED PROJECTS APPROVAL FORM

SEND NOTICE OF AWARD TO:
The University of Florida
Division of Sponsored Research
219 Grinter Hall
Gainesville, FL 32611
(904) 392-1582

AGENCY APPLICATION DEADLINE
(DO NOT LEAVE BLANK)

Date: March 18, 1991

☒ **postmark** ☐ **receipt**

9K University Project # 91031509
(LEAVE BLANK)

Title of Proposal: Development of Transgenic C_3 Plants Requiring Less ATP for NH_4^+ Assimilation

Submitted to Agency/Program: USDA NRI, Competitive Grants Program
(NOTE TO THE P.I.: Please provide mailing instructions on page 2)

UNIVERSITY ENDORSEMENTS: The attached proposal has been examined by the officials whose signatures appear below. The principal academic review of the proposal is the responsibility of the **Department/Center and College.** If additional space is needed for signatures, please provide them on a separate sheet of paper.

Principal Investigator: (Project Director)

NAME: Robert R. Schmidt
Date
TITLE: Graduate Research Professor
CAMPUS ADDRESS: 3103 McCarty Hall
TELEPHONE: (904) 392-0237
SOC. SEC. NO. 229-38-8422

Approval by Dean or Director: (If more than one)

3/14/91
NAME: N. P. Thompson
Date
TITLE: Assoc. Dean for Research

~~Co~~-Principal Investigator: (If Applicable)

Robert R Schmidt 3-13-91
NAME:
Date
TITLE:
TELEPHONE:
SOC. SEC. NO.

Other Endorsement (If Needed):

NAME:
Date
TITLE:

Department Head:

E M Hoffmann 3/13/91
NAME: Edward M. Hoffmann
Date
TITLE: Professor and Chairman

Approval by Vice-President for Agricultural Affairs
(For all projects involving IFAS Personnel)

Carol A Cook 3/14/91
NAME: Carol A. Cook, Assistant Director
Date
TITLE: IFAS Sponsored Programs

Department Head: (If more than one)

NAME:
Date
TITLE:

Approval by Vice-President for Health Affairs:
(For all projects involving JHMHC Personnel)

NAME:
Date
TITLE:

Approval by Dean or Director:

NAME:
Date
TITLE:

Official Authorized to Sign for the University:
(Leave Blank)

03/18/91
NAME: Dillard C. Marshall
Date
TITLE: Assistant Director of Research
Division of Sponsored Research
University of Florida

DSR-1 (6/88)

TO BE COMPLETED BY PRINCIPAL INVESTIGATOR
(Do Not Complete Shaded Boxes)

PRINCIPAL INVESTIGATOR

Schmidt — Last Name (Print or Type)
R R — Initials
229-38-8422 — Social Security Number

Microbiology & Cell Science — Department or Unit to Administer Account — MCN
IFAS/Agriculture — College — IF

CO-PRINCIPAL INVESTIGATOR

Last Name (Print or Type)
Initials
Social Security Number
Department
College

Title of Project: Development of Transgenic C_3 Plants Requiring Less ATP for NH_4^+ Assimilation

INDIRECT COST (IDC):

EIES AND IFAS FACULTY OMIT THIS SECTION.

FOR ALL APPLICABLE IDC ACCRUED, RETURN IDC TO: (CHECK ONE)

XX (A) PI and Home Department Only.
___ (B) PI, CO-PI and their Home Departments (equal split).
___ (C) PI, Center and Home Department.
___ (D) PI and Center Only (No Department IDC return).
___ (E) OTHER (provide explanation) ___

[X]

NAME OF DEPARTMENT(S) TO ACCRUE IDC, IF APPLICABLE ___

NAME OF CENTER TO ACCRUE IDC, IF APPLICABLE ___

IF SPONSOR DOES NOT ALLOW IDC, PLEASE CHECK HERE: [ ]

CERTIFICATIONS AND ASSURANCES

		Exp Date	Number
Human Subjects	[ ]		
Animal Subjects	[ ]		
Recombinant DNA/RNA	[XX]		
FDA Good Lab Practices	[ ]		
Biohazards	[ ]		

Communication Component (Pub/Audio Visual) IFAS Only [ ]

(If none, please check here): [ ]

Proprietary Data	[ ]
Copyright	[ ]
Invention	[ ]
Clearinghouse Review	[ ]
Classified Research	[ ]

TYPE:
- New [ ]
- Renewal [X]
- Continuation [ ]
- Supplemental [ ]
- Revised [ ]

CATEGORY:
- Research [X]
- Training [ ]
- Other [ ]

MAILING INSTRUCTIONS:

Mail Original and 14 copies to: (Check One)
National Research Inititative Competitive Grants Program, c/o Proposal Services Branch, CSRS, USDA, Room 303 Aero-space Centr, Washington DC 20250-2200

- [ ] First Class
- [ ] Federal Express
- Samas Acc't. # ___
- [ ] Other ___

Send 2 copies to P.I.

DSR USE ONLY

UPN: 91031509 PROPOSAL DATE: 031591 AGENCY CODE: F001

AGENCY: US Dept of Agriculture

DIVISION:

BUDG BEG: 090191 BUDG END: 083192 PROJ BEG: 090191 PROJ END: 083192

AMT REQUESTED: 69540

INDIRECT COSTS—AMT $: 8540 RATE: 140 BASE: TDC

OFF CAMPUS— $: RATE: BASE:

COST SHARING— AMT $: COST SHARING %:

RECEIVED: ___ BUDGET CHECK: KM 1st CLS: 3-18-91 FEDEX: ___ DIRECT: ___ 205: ___

UFFL: ___ YES ___ NO DATE CONTACTED: ___ NR: ___ INTERNAL: ___ COPIES NEEDED: ___

DSR-1 (6/88)

UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH SERVICE

GRANT APPLICATION

OMB Approved 0524-0022
Expires 8/92

FOR CSRS USE ONLY	
Program Area Code	*Proposal Code*

1. Legal Name of Organization to Which Award Should be Made
University of Florida - IFAS

3. Name of Authorized Organizational Representative

4. Telephone Number (Include Area Code)
(904) 392-1582

2. Address (Give complete mailing address and Zip Code-including County)
Division of Sponsored Research
219 Grinter Hall
University of Florida
Gainesville, FL 32611

5. Address of Authorized Organizational Representative (If different from Item 2.)
Same as item 2

6. Title of Proposal (80-character Maximum, including spaces)
Development of Transgenic C_3 Plants Requiring Less ATP for NH_4^+ Assimilation

7. Program to Which You are Applying (Refer to Federal Register Announcement where applicable) National Research Initiative Competitive Grants Program

8. Program Area and Number (Refer to Federal Register Announcement where applicable)
Nitrogen Fixation 54.2

9. IRS No.	10. Congressional District No.	11. Period of Proposed Project Dates	12. Duration Requested
59-6002052	Sixth District	From: 9/1/91 Through: 8/31/94	36 months

13. Type of Request
☐ New ☐ Resubmission ☒ Renewal ☐ Supplement ☐ Continuing Grant Increment
☐ PI Transfer ☐ (of/to USDA Grant No. ______) ☐ Other

14. Funds Requested (From Form CSRS-55)
$217,835

15. Principal Investigator(s)/Project Director(s)
a. PI/PD #1 Name (First, Middle, Last) and Social Security Number* (Correspondent PI)
Robert R. Schmidt 229-38-8422

b. PI/PD #2 Name (First, Middle, Last) and Social Security Number*

c. PI/PD #3 Name (First, Middle, Last) and Social Security Number*

16. PI/PD #1 Phone Number (Include Area Code)
(904(392-0237

17. PI/PD #1 Business Address (Include Department/Zip Code)
Department of Microbiology and Cell Science
3103 McCarty Hall
University of Florida
Gainesville, FL 32611

Submission of the Social Security Number is voluntary and will not affect the organization's eligibility for an award. However, it is an integral part of the CSRS information system and will assist in the processing of the proposal.

18. Type of Performing Organization (Check one only)
01 ☐ USDA/S&E Laboratory
02 ☐ Other Federal Research Laboratory
03 ☐ State Agricultural Experiment Station (SAES)
04 ☒ Land-Grant University 1862
05 ☐ Land-Grant University 1890 or Tuskegee University
06 ☐ Private University or College
07 ☐ Public University or College (Non Land-Grant)
08 ☐ Private Profit-making
09 ☐ Private Non-profit
10 ☐ State or Local Government
11 ☐ Veterinary School or College
12 ☐ Other (Specify)

19. Will the Work in This Proposal Involve Recombinant DNA?
☐ No ☒ Yes (If yes, complete Form CSRS-662)

20. Will the Work in This Proposal Involve Living Vertebrate Animals?
☒ No ☐ Yes (If yes, complete Form CSRS-662)

21. Will the Work in This Proposal Involve Human Subjects?
☒ No ☐ Yes (If yes, complete Form CSRS-662)

22. Will This Proposal be Sent or has it Been Sent to Other Funding Agencies, Including Other USDA Agencies?
☐ No ☒ Yes (If yes, list Agency acronym(s) & program(s)) NSF

By signing and submitting this proposal, the prospective grantee is providing the required certifications set forth in 7 CFR Part 3017, as amended, regarding Debarment and Suspension and Drug-Free Workplace; and 7 CFR Part 3018 regarding Lobbying. **Submission of the individual forms is not required.** (Please read the Certifications and Instructions included in this kit before signing this form.)

In addition, the prospective grantee certifies that the information contained herein is true and complete to the best of its knowledge and accepts as to any grant award, the obligation to comply with the terms and conditions of Cooperative State Research Service in effect at the time of the award.

Signature of Principal Investigator(s)/Project Director(s) (*All PI's/PD's* listed in block 15 must sign if they are to be included in award document.)
Robert R Schmidt

Date
3/12/91

Signature of Authorized Organizational Representative (Same as Item 3)

Title

Date

Form CSRS-661

TABLE OF CONTENTS

PROJECT SUMMARY

Under photorespiratory conditions, C_3 plants expend a large amount of ATP/reducing equivalents for net assimilation and reassimilation of NH_4^+ by the chloroplastic glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. Chemical mutagenesis and Agrobacterium-mediated transformation of Arabidopsis will be used to replace its GS/GOGAT pathway with a Chlorella chloroplastic glutamate dehydrogenase (α-NADP-GDH) which has a high NH_4^+ affinity. The cDNA and nuclear gene, encoding the precursor-protein for this enzyme, will be inserted into a binary vector for transformation of root explants which will be regenerated into plants. NADP-GDH cDNA/gene constructs will be placed under the control of homologous or heterologous promoters. Chloroplast transit-peptides from Chlorella and Arabidopsis will be tested for their abilities to direct the import of the precursor-protein into Arabidopsis chloroplasts. Constructs are also designed to determine if the precursor-mRNA transcript from the NADP-GDH gene (with many introns) will be processed correctly. Transgenic plants expressing NADP-GDH activity will be analyzed to ascertain whether an increase in efficiency of NH_4^+ assimilation is translated into a net gain in plant productivity.

PROJECT DESCRIPTION

A. Objectives

1. To use a combination of chemical mutagenesis and Agrobacterium-mediated transformation of Arabidopsis thaliana to replace its chloroplastic glutamine synthetase/glutamate synthase (GS/GOGAT) pathway with the Chlorella sorokiniana chloroplastic NADP-specific glutamate dehydrogenase (NADP-GDH) α-homohexamer which has a very high affinity for NH_4^+.

2. To determine whether this pathway replacement will improve the energy efficiency of NH_4^+ assimilation/reassimilation in a C_3 plant under photorespiratory conditions by saving one ATP for every NH_4^+ incorporated into glutamate.

3. To provide comparative biochemistry/molecular biology data on the ability of gene/cDNA (and associated intron splice sites, regulatory regions, etc.) and the chloroplast transit peptide sequence from a lower eukaryotic plant cell to be expressed or function in a higher plant.

B. Background, Significance, and Progress Report

In chloroplasts of C_3 higher plants (e.g., Arabidopsis) GS and ferredoxin-dependent GOGAT function together in the biosynthesis of glutamate from NH_4^+ produced (i) by reduction of NO_3^- absorbed from the soil, and (ii) from deamination/decarboxylation of glycine during photorespiration (Fig. 1A). The primary route of inorganic nitrogen into organic nitrogen metabolism in higher plants is via transamination of the α-amino group of glutamate (synthesized by the GS/GOGAT pathway) to α-keto acids (1,2). Ammonium assimilation and reassimilation require a very large expenditure of ATP and reducing equivalents, particularly under photorespiratory conditions (3-5). This ATP expenditure becomes even greater as the nitrogen nutrition of the plant is increased. We have observed (unpublished data) that total GS activity in certain C_4 grasses can increase more than three-fold as the concentration of NO_3^- or NH_4^+ in the nutrient medium is increased from 1 mM to 20 mM.

There appears to be a major difference between some lower organisms and higher plants in how they regulate NH_4^+ assimilation and the utilization of ATP for this process, particularly at high nitrogen levels. At high NH_4^+ levels, many bacteria, fungi, and green algae repress/inhibit the GS/GOGAT pathway and induce a NADP-GDH (6,7) to incorporate NH_4^+ into organic nitrogen metabolism via glutamate (Fig. 1B). By use of this alternate route instead of the GS/GOGAT pathway, one ATP is saved for every glutamate synthesized, thereby making nitrogen assimilation more energy efficient at high nitrogen levels. Most higher plants do not have an NH_4^+ inducible NADP-GDH and therefore assimilate NH_4^+ by the GS/GOGAT pathway regardless of the level of nitrogen nutrition. Although most plants have a constitutive mitochondrial NAD-GDH and some have a chloroplastic NAD(P)-GDH, these GDHs (7) have low affinities for NH_4^+ (K_m of 5-50 mM) compared to the plant GS isoenzymes which have very high affinities for NH_4^+ (K_m of 0.01-0.02 mM). One experimental approach for possibly increasing the efficiency of NH_4^+ assimilation/reassimilation in higher plants is to replace the chloroplastic GS/GOGAT pathway with a high affinity NADP-GDH from a lower

organism.

Research in this laboratory has revealed that the unicellular green alga, Chlorella sorokiniana, has a constitutive, mitochondrial, tetrameric NAD-GDH (identical subunits, each 45,000 Da) and two NH_4^+-inducible chloroplastic, homohexameric NADP-GDH α- and β-isoenzymes (subunits 55,500 or 53,000 Da, respectively) which have strikingly different affinities for NH_4^+ (8-11). We have determined (11) that below 3 mM NH_4^+ in the culture medium only the α-homohexamer accumulates in the chloroplast. Above this NH_4^+ concentration, both isoenzymes initially accumulate (i.e., 1st 120 min), then accumulation of the α-subunit ceases, and only the β-homohexamer continues to accumulate at long induction times (i.e., 3-12 h). From additional nitrogen nutrition studies in which the cells were subjected to rapid transitions between low and high NH_4^+ concentrations, we concluded (11,13) that some type of feedback mechanism switches gene expression from synthesis of the α-subunit to the β-subunit as intracellular nitrogen metabolite(s) reach a certain threshold concentration.

The C. sorokiniana α-homohexamer has a very high affinity for NH_4^+ (K_m ranges between 0.02 mM and 3.5 mM) and is allosteric in that its NH_4^+ K_m varies with NADPH concentration (11). Our serach for the scientific literature has not revealed any other reports of a GDH with an NH_4^+ K_m as low as 0.02 mM. This NH_4^+ K_m falls into the range of those reported for higher plant GS isoenzymes (0.01 mM - 0.02 mM). In contrast, the β-homohexamer has a low affinity for NH_4^+ (K_m = 75 mM) and is non-allosteric with respect to NADPH. When cells are synthesizing both α- and β-subunits early during the induction period at high ammonium concentration, homo- and heterohexamers (i.e., 6α, $5\alpha{:}1\beta$, $4\alpha{:}2\beta$, $3\alpha{:}3\beta$, $2\alpha{:}4\beta$, $1\alpha{:}5\beta$, and 6β) accumulate within the chloroplast (37). These NADP-GDH heterohexamers presumably have NH_4^+ K_m values which fall between those of the two homohexamers (11). In addition to the process which controls the differential synthesis of the α- and β-subunits, we have shown (20,21) that the levels/activities of the NADP-GDH holoenzymes are regulated by a Ca^{+2} and ATP dependent inactivation/degradation process involving covalent-modification of the subunits as an obligatory step to their degradation.

By use of a very specific polysome immunoselection procedure, coupled with oligo(dT) chromatography, we purified the NADP-GDH mRNA 1290-fold to apparent homogeneity from C. sorokiniana cells accumulating primarily the β-homohexamer (35). In vitro translation of this purified mRNA produced a single protein with a molecular weight of 58,500 (35). In vitro translation of total poly(A)$^+$RNA, isolated from cells synthesizing primarily the α- or β-homohexamer resulted in the synthesis of 58,500 Da precursor-protein(s) which are processed in vitro (by C. sorokiniana extracts) to 55,500 Da and 53,000 Da subunits (11,36). These two subunits have very similar peptide maps, and both can be immunoprecipitated by polyclonal antibodies prepared against one of the subunits, indicating that they have a high degree of sequence homology (11). This sequence homology between the α- and β-subunits and the identical size of their precursor-proteins prompted us to consider whether these two subunits might arise from (i) the differential processing of a precursor-protein encoded by a single mRNA and gene, (ii) the specific processing of two very similar precursor-proteins encoded by two mRNAs formed by alternative splicing (16,17) of a precursor mRNA transcribed from a single gene, or (iii) the specific processing of two precursor-proteins encoded by two

mRNAs transcribed from two closely related genes.

Progress Report (September 1, 1989 - March 1, 1991)

The previous grant proposal "Nuclear Gene Encoding Two NH_3-Inducible Chloroplastic Isoenzymes" listed 6 research objectives and requested $313,236 for three years. The actual award (Agreement No. 89-37262-4843) was $90,000 for two years beginning September 1, 1989. Because the amount and time of the award were decreased by approximately 70% and 30%, respectively, approval was given to decrease the number of objectives from six to four:

1. To elucidate the molecular mechanisms regulating the differential accumulation of two chloroplast-localized NH_4^+-inducible α- and β-NADP-GDH isoenzymes which *appear* to be encoded by the same nuclear gene (i.e., establish unequivocally the number of NADP-GDH genes).

2. To determine how many mRNAs encode the NADP-GDH α- and β-isoenzymes.

3. To measure the kinetics of accumulation/degradation of the NADP-GDH mRNA(s).

4. To determine if the α- and β-subunits are formed by differential processing of the same precursor-protein.

During the first 18 mo. of this 24 mo. grant period, our research progress has been excellent in that all or major portions of the first three objectives have been accomplished and the fourth should be completed by the end of the grant period (8/31/91). The details of our research progress are presented in the two manuscripts (submitted in March 1991 to PLANT MOLECULAR BIOLOGY) which are in the Appendix of this proposal. A summary progress report is presented below:

A cDNA library was prepared from total poly(A)$^+$RNA extracted from C. sorokiniana cells which were simultaneously synthesizing the NADP-GDH α- and β-subunits. Seventeen independent NADP-GDH cDNA clones were isolated. A comparison of their restriction maps and nucleotide sequences indicated that all of them were derived from a single mRNA species. From these clones, a 2,145 bp consensus sequence was derived which contains a 1,571 bp open-reading-frame (ORF) which encodes a 57,401 Da protein approximately 98% of the size (58,500 Da) of the precursor-protein(s) from which the subunits are processed. Although the consensus cDNA did not contain the ATG start codon, it encoded part of the chloroplast transit-peptide sequence and sufficient sequence for the 55,500 Da α-subunit and 53,000 Da β-subunit. The deduced amino acid sequence of the C. sorokiniana NADP-GDH cDNA is 50% and 50.3% identical with those of the Escherichia coli (39) and Neurospora crassa (40), respectively, for their entire lengths which overlap. However, comparison of the sequences of the highly conserved region identified by Mattaj et al. (41) showed much stronger homologies of 76.6% and 73.4% respectively.

Analysis of codon usage in the C. sorokiniana NADP-GDH encoding ORF revealed a strong bias towards the use of codons containing G and C at both the first and third

positions. This preference correlated with the high GC content (63%) for C. sorokiniana genomic DNA that we reported earlier (12). Furthermore, for most amino acids, there was an extreme preference for a particular base at the third position of the codon (i.e., G for leucine and valine codons and C for serine, proline, threonine, arginine and glycine codons). Expressed ribulose bisphosphate carboxylase/oxygenase small subunit genes of Chlamydomonas reinhardtii (42) exhibit the same preference for the codons most frequently used in the Chlorella NADP-GDH gene. Genes of monocotyledonous plants also show a preference for codons containing G or C as the third degenerate base (43,44). Although the codons utilized most frequently in the Chlorella NADP-GDH also occur at a higher frequency in genes of higher organisms than in bacteria (45), we fused the longest (1.91 bp) C. sorokiniana NADP-GDH cDNA in frame with lac z in a Bluescript vector and showed it to be expressed under the control of the lac promoter as both antigen and activity in E. coli. This apparent flexibiity in codon usage suggests that this cDNA may be expressed as active NADP-GDH activity in higher plants.

During a 240 min induction period, under conditions in which both types of subunits were synthesized in C. sorokiniana, the kinetics of NADP-GDH mRNA accumulation were measured by Northern blot analysis, using cDNA probes corresponding to the highly conserved region or the 3′-untralslated region from the consensus cDNA. The rationale was that the highly conserved region probe should hybridize to any NADP-GDH mRNA which has this conserved region in common whereas the 3′-untranslated region should hybridize only to the unique mRNA from which it was derived. Both probes detected only a single-size mRNA (2.2 kb) and yielded the same pattern of mRNA accumulation throughout the induction period. The pattern of mRNA accumulation indicated that both transcription and degradation regulate the level of this mRNA. After a 20 min induction lag, the concentration of NADP-GDH mRNA (per ml of culture or as % of total poly(A)$^+$RNA) rapidly increased 16-fold reaching a maximum between 60 and 80 min. A net loss in NADP-GDH mRNA (~30%) occurred between 80 and 100 min and then its concentration increased again but more slowly between 120 and 240 min. The sharp oscillation in NADP-GDH mRNA concentration resulted in only small to moderate changes in the rate of accumulation of total NADP-GDH catalytic activity. This type of discrepancy between the two patterns suggests that some type of mRNA translational control and/or enzyme covalent-modification/turnover is preventing expression of the total hybridizable NADP-GDH mRNA as the accumulation of a proportional amount of total NADP-GDH activity.

The aforementioned cDNA isolation/sequencing data along with the results from the Northern blots analyses with the two cDNA probes, are consistent with C. sorokiniana having only a single mRNA species that is translated into a NADP-GDH precursor-protein which is differentially processed to yield α- or β-subunits (15).

The highly conserved region probe was used to select eight NADP-GDH genomic clones from a C. sorokiniana genomic library. Restriction maps of the four longest overlapping genomic clones showed them to span a 21.9 kb region of the genome. Eleven kilobases of this region were sequenced and shown to contain the complete 2,145 bp NADP-GDH cDNA consensus sequence distributed among 22 exons. The exons ranged in size from 18 bp (the smallest reported for any plant) to 550 bp. The 21

introns is unusual in that genes from eukaryotic microorganisms are reported to contain on the average only 1 to 3 introns.

To determine whether the sequence derived from NADP-GDH genomic clones corresponds to the only NADP-GDH gene in C. sorokiniana, Southern blot analyses were performed on restriction fragments produced by several endonucleases on the genomic clones and on the total cellular genomic DNA. The restriction fragments were hybridized to probes from the highly conserved region and the 3'-untranslational region, again the rationale being that the highly conserved region probe should hybridize to any NADP-GDH gene in the C. sorokiniana genome whereas the other probe to only the unique NADP-GDH gene having that specific region. The Southern blot data clearly showed that the NADP-GDH gene, which we isolated and sequenced, is the only NADP-GDH gene having the highly conserved region. Very convincing evidence came from a SmaI digest of the total cellular genomic DNA that produced only a single 6.9 kb fragment which hybridized with both probes. This endonuclease also was shown to produce a 6.9 kb restriction fragment (containing the regions of hybridization with both probes) from the NADP-GDH gene which we sequenced. Thus, a single C. sorokiniana NADP-GDH gene encodes the sequence for the α- and β-subunits which can be assembled into holoenzymes with strikingly different K_m values for ammonium (14).

We currently have experiments in progress related to the fourth objective which should be completed by the end of this grant period (8/31/91). The aforementioned molecular biology experiments have shown that it is very likely that a single precursor-protein is differentially processed to form the α- and β-subunits. However, we want to demonstrate in vitro that a single precursor-protein, synthesized in vitro from a full-length cDNA in an expression vector system, can be differentially processed using extracts from Chlorella cells synthesizing only the α- or β-subunit. Moreover, we are currently purifying two preparations of the α- and β-subunits to determine their N-terminal amino acid sequences so that the cleavage sites in the precursor-proteins can be positioned. This latter information is required so that the endopeptidase cleavage-site which yields the β-subunit can be modified by in vitro mutagenesis so that only the α-subunit cleavage site remains. For many of the plant biotechnology experiments described in this new proposal, we will want only the α-subunit with its high affinity for NH_4^+, to be expressed from the various cDNA/gene contructs in Arabidopsis. However, the unmodified cDNA/gene also will be used in comparative biochemistry/molecular biology studies.

C. Experimental Plan and Methods

Selection of Arabidopsis mutant(s) having both low GS activity and absence of GOGAT activity

Arabidopsis (22) and barley (23) mutants have been isolated which are deficient in GOGAT or chloroplastic GS activities, respectively. These mutants were selected for their ability to grow in atmospheres with elevated CO_2 levels (0.8 - 1.0%) but not in normal air. The basis of the mutant selection was that CO_2 competitively inhibits the oxygenase activity of ribulose bisphosphate carboxylase/oxygenase (Rubisco) which catalyzes the formation of phosphoglycollate, the first intermediate on the photorespiratory pathway. Because of the importance of both the chloroplastic GS and

GOGAT in the reassimilation of NH_4^+ produced during photorespiration, a deficiency in either of these enzymes leads to accumulation of NH_4^+ in the leaves and rapid inhibition of photosynthesis after these mutants are transferred to air in the light. Under these photorespiratory conditions, the mutants become chlorotic within several days, and can be rescued by returning them to an elevated CO_2 atmosphere in the light. Since plants containing mutations in genes encoding some of the other enzymes in the photorespiratory pathway may also give the chlorotic phenotype under photorespiratory conditions, direct enzyme analysis (22) of leaf extracts is required to identify specific GS and GOGAT mutants.

In leaves of wild-type C_3 plants, such as barley and Arabidopsis, the chloroplastic GS isoenzyme has been shown (24) to represent a much higher percentage of the total GS activity than the cytosolic GS (approx. 85:15, respectively). The higher plant cytosolic and chloroplastic GS isoenzymes are encoded by different nuclear genes (25-28). Wallsgrove et al (23) isolated a barley mutant deficient in the chloroplastic GS but which still contained wild-type levels (i.e., approx. 17% of total GS activity in leaves) of the cytosolic GS. Under elevated CO_2 levels in the light, this barley mutant grew normally, indicating that the remaining wild-type activity of the cytosolic GS was sufficient to meet the glutamine requirement of the plant for biosynthesis of purines, pyrimidines, arginine, histidine, and tryptophan. However, when the mutant plant was placed under photorespiratory conditions, the cytosolic GS by itself was unable to reassimilate the large amount of NH_4^+ produced during photorespiration.

Although an Arabidopsis thaliana (Columbia ecotype) GOGAT mutant (GluS) has been isolated by Somerville and Ogren (22), neither cytosolic nor chloroplastic GS mutants have been isolated yet for this plant. Dr. C. Somerville (Michigan State University) has given us seed of his GOGAT mutant (GluS; MSU 254) for use in this project. However, we will have to isolate Arabidopsis mutants which contain wild-type cytosolic GS activity and are deficient (0-20%) in chloroplastic GS activity. When these chloroplastic GS mutants (homozygous) are isolated, they will be crossed with the GOGAT mutant. From the resulting progeny, a double-mutant homozygous for both the GOGAT and chloroplastic GS mutations will be isolated. This double mutant is required for the development of a transgenic plant in which the biosynthesis of glutamate in the chloroplast will occur via the NADP-GDH α-homohexamer (introduced from Chlorella) instead of the GS/GOGAT pathway (Fig. 1C). Somerville and Ogren (22) observed that, in the Arabidopsis GOGAT mutant under photorespiratory conditions, the chloroplastic GS rapidly converted free glutamate to glutamine, resulting in the deprivation of free glutamate for use in biosynthesis of the other amino acids. Thus, unless the wild-type level of the chloroplastic GS is low or absent in the transgenic plant, the glutamate synthesized by the NADP-GDH may be rapidly converted to glutamine, resulting in a shrinkage in the pool of available glutamate normally used in transaminase reactions. It should be noted that the primary route for assimilation of inorganic-nitrogen into organic nitrogen metabolism is via transamination of the α-amino group of glutamate into the carbon skeletons of amino acid precursors.

We will select Arabidopsis GS mutants by the same procedure described by Somerville and Ogren (22) and Estelle and Somerville (29) to isolate their GOGAT mutant (GluS, MSU 254). Mutagenesis will be accomplished by soaking seeds in a 0.3% solution of ethyl methane sulfonate. This treatment will induce heterozygous mutations in some of the cells which will give rise to the reproductive structure of the plant. This

M1 generation will be cultured to maturity in normal air under fluorescent lamps, allowed to self-fertilize, and the seed will be collected. The seed will be germinated at high densities in the light under an atmosphere of 1% CO_2-air, and these M2 progeny will be screened by placing them into normal air for 3-4 days. The plants which show chlorosis will be identified and returned to the high-CO_2 environmental growth chamber and allowed to self-fertilize and produce seed. The seed from each M2 plant then will be germinated separately in the high-CO_2 atmosphere, transferred to normal-air to identify homozygous mutant progeny, and then returned to the high-CO_2 atmosphere for recovery and further growth. After a suitable recovery time from chlorosis, extracts will be prepared from the leaves of these M3 progeny and will be analyzed for total GS activity. When extracts having low total GS activity are identified, these will be further analyzed by ion-exchange chromatography in a Pharmacia FPLC (analytical Mono Q column, NaCl gradient) to determine the ratio of activities of the cytosolic and chloroplastic GS isoenzymes. Those mutants which have a wild-type level of the cytosolic GS, and are deficient (0-20%) the chloroplastic GS, will be allowed to self-fertilize and their seed will be collected. Progeny from these seed will be used in crosses with the GOGAT mutant to produce the chloroplastic GS/GOGAT double-mutants as discussed above.

Agrobacterium-mediated transformation of Arabidopsis

Several types of binary Ti plasmid vectors have been used for the Agrobacterium-mediated transformation of the different ecotypes of Arabidopsis (30-34). In addition to their ability to replicate in both Agrobacterium and E. coli, these binary vectors usually have both left and right border repeats of the T-DNA region, a dominant marker gene (e.g., kanamycin or hygromycin B resistance), several unique restriction sites for insertion of foreign DNA between the T-DNA borders, and an antibiotic gene for maintenance in the resident bacterium (30). For the T-DNA region of the binary vector to be transferred into a plant cell, the Agrobacterium strain must also carry a helper Ti plasmid which provides the necessary transacting vir functions which are lacking in the binary vector. To prevent recombination with binary vectors, helper plasmids have had their T-DNA region deleted.

Binary-vectors carrying the genes for kanamycin or hygromycin B resistance appear to vary in their effectiveness as selectable markers for use in isolation of transformed cells from the various Arabidopsis ecotypes. For example, the Columbia ecotype is reported (31) to have some natural resistance to kanamycin whereas Wassilskija and Landsberg erecta are very sensitive to this antibiotic. For those ecotypes with some resistance to kanamycin, hygromycin B has proven to be an effective selectable marker. Feldmann and Marks (32) and Feldmann et al. (33) have successfully transformed Arabidopsis (ecotype Wassilskija) by germinating seeds in the presence of Agrobacterium carrying a kanamycin resistant binary vector. Although this transformation method appears to be very simple and convenient, there are reports (personal communication with various scientists) that transformation frequencies are often low and vary among different seed lots. Lloyd et al. (31) have transformed Arabidopsis (Columbia ecotype) by a modified leaf-disk transformation/regeneration method using a binary vector encoding hygromycin B rather than kanamycin. Because uninfected control leaf-tissue of the Columbia ecotype also developed callus in the presence of kanamycin, this antibiotic was ineffective as a selecting agent with this ecotype in the leaf-disk method.

However, with the hygromycin-resistant vector, approximately one-third of the original transformed leaf pieces survived the hygromycin selection-step and more than 50% of these generated shoots. Four months were required from the time of infection of leaf-tissue pieces with Agrobacterium until the collection of seed from the transformed plants. Valvekens et al. (34) have developed cultural conditions for inducing root-explants, of several Arabidopsis ecotypes, to generate shoots rapidly and at 100% efficiency. By use of this root-explant regeneration procedure, along with a Ti plasmid vector encoding kanamycin resistance, transformed seed-producing plants were obtained with an efficiency of 20-80% within 3-months after gene transfer. In addition to a shorter time to obtain seed from transformed tissue, this root explant transformation/regeneration method which employed kanamycin was successful with three different ecotypes (i.e., Columbia, Landberg erecta, and C24). This finding contrasts with the reported difficulties of the Columbia ecotype by the leaf-disk method discussed above (31).

Because the Columbia ecotype was used to select the chloroplastic GOGAT mutant, which was obtained from Dr. Somerville, we initially plan to use the root explant transformation/regeneration method of Valvekens et al. (34) and a Ti plasmid binary vector carrying the kanamycin resistance gene. The binary vector system (GUS Gene Fusion Kit) will be purchased from Clontech Laboratories. This system utilizes A. tumefaciens strain LBA4404 with its helper plasmid based on an octopine Ti plasmid, and several modified binary vectors: plasmid pBI101 (GUS cassette, no promoter), plasmid pBI121 (pBI101 with CaMV 35S promoter, and plasmid BI221 (pBI121 GUS cassette in pUC19). The kit also contains the conjugative plasmid RK2013 in HB101. The aforementioned binary vectors contain, between the right and left borders, the kanamycin resistance gene (npt II) which is driven and terminated by the nopaline synthase (NOS) promoter (NOS-pro) and terminator (NOS-ter), respectively. The β-glucuronidase (GUS) gene in pBI121 is driven and terminated by the CaMV 35S promoter and the NOS-ter, respectively. The 3′ and 5′ termini of the CaMV 35S promoter and NOS-ter termini, respectively, have unique restriction sites which will permit excision of the GUS gene and its replacement with the Chlorella NADP-GDH cDNA or genomic DNA. To determine if the natural promoter of the Chlorella NADP-GDH gene can be expressed (without or with in vitro mutagenesis) in Arabidopsis, the "promoter-less" GUS cassette in pBI101 will be used. In this binary plasmid, the CaMV 35S promoter has been deleted and a multicloning site has been inserted in its place 5' to the GUS gene. Thus, various promoters (e.g., NADP-GDH promoter region) can be cloned upstream of GUS which can be used as a reporter gene.

Analysis of expression of Chlorella NADP-GDH cDNA/genomic DNA in transgenic Arabidopsis plants

The Arabidopsis GS/GOGAT mutant will be transformed with the aforementioned binary vector(s) carrying a number of different Chlorella NADP-GDH cDNA/genomic DNA constructs:

a. Full-length NADP-GDH cDNA carrying its own ATG start-codon, chloroplast transit-peptide sequence, and its 3′-terminus devoid of its poly(A)tail (i.e., the Nos-ter will provide the terminator/polyadenylation signal).
b. The same cDNA (as a.) modified by replacement of the Chlorella chloroplast transit-peptide sequence with the equivalent higher plant sequence reported (38) for one of

the four Arabidopsis Rubisco small subunit precursor-proteins (e.g., standard single-letter code for amino acids for transit-peptide AtB is MASSMLSSAAVVTSPAQATMVAPTGLKSSASFPVTRKANNDITSITSNGGRV SC). Alternatively, we will be screening an Arabidopsis cDNA library with a heterologous GS cDNA probe (Phaseolus vulgaris) to isolate the chloroplastic GS ~~cDNA. In the event that the import and/or processing of precursor-proteins for stromal enzymes, involved in different aspects of chloroplast metabolism (e.g., carbon~~ vs. nitrogen) is/are regulated in part by transit-peptides with sequences differences, it might be advantageous to use the transit-peptide sequence for the Arabidopsis chloroplastic GS instead of the one for the Rubisco small subunit.

c. The same cDNA (as a.) without any chloroplast transit peptide sequence (i.e., the NADP-GDH will be targeted for the cytosol instead of the chloroplast).
d. The entire NADP-GDH genomic DNA clone (gene) containing its natural promoter region, start codon, exons, introns, and termination/polyadenylation signal(s).
e. ~~Another related construct will be the NADP-GDH~~ promoter region by itself.
f. ~~The same NADP-GDH gene (as d.) modified by deletion of its natural promoter~~ region.

The a., b., c., and f. constructs will be inserted into vector pBI121 between CaMV 35S promoter and Nos-ter to replace the deleted GUS gene. The d. construct will be inserted into the "promoter-less" vector pBI101 to replace its GUS gene. The e. construct will be inserted into the "promoter-less" vector pBI101 in front of the GUS gene to determine whether the Chlorella promoter will be able to drive the GUS gene. There are undoubtedly other constructs that will be required; however, the aforementioned should serve as examples of some of the more important constructs.

Transformants from the root-explants initially will be identified by their kanamycin resistance, and regenerant plants will be allowed to self-fertilize and produce seed in a high-CO_2 lighted, environmental chamber. These seed will be germinated in the presence of kanamycin in a high CO_2 atmosphere and the antibiotic resistant progeny will be identified. These will be placed under photorespiratory conditions (light, normal air) to identify which plants remain green (if any) and those which become chlorotic. The plants which remain green will be allowed to self-fertilize and produce seed under photorespiratory conditions whereas the chlorotic plants will be returned to the high CO_2 atmosphere for seed production. The seed from these plants will be germinated in the low or high CO_2 atmospheres and extracts of their leaves will be analyzed for NADP-GDH activity (spectrophotometrically), NADP-GDH anti-gen (Western blotting), NADP-GDH mRNA (Northern blotting) and NADP-GDH DNA (Southern blotting). Assays will also be performed for total GS activity to verify that transformation did not alter the wild-type level of the cytosolic GS in the transgenic plant. To confirm that kanamycin resistance is conferred by neomycin phosphotransferase and not by some other mechanism in the transformants, assays for this activity will also be performed. The aforementioned assays for NADP-GDH-antigen, -mRNA, and -DNA will be particularly important for transgenic plants which become chlorotic under photorespiratory conditions. For example, if the Arabidopsis genome contains the intact NADP-GDH cDNA/gene and the plant does not accumulate active enzyme, it might be possible to identify the biochemical step (i.e., transcription, translation, post-translation) that is

limiting the accumulation of active NADP-GDH. If the transgenic plants (green or chlorotic) contain NADP-GDH antigen or activity, their chloroplasts will be isolated and analyzed to ascertain whether the antigen/activity is chloroplast localized. For the plants transformed with NADP-GDH cDNA without a chloroplast transit-peptide sequence, assays will be performed to show whether or not the NADP-GDH is accumulating in the cytosol. From a comparative biochemical/molecular biology viewpoint, the results from the aforementioned assays on the transgenic plants, carrying the different cDNA/gene constructs, are important for identifying possible differences in gene-enzyme regulation (or processing) in higher and lower plants. For example, from the cDNA constructs having the Chlorella or higher-plant chloroplast transit-peptide sequence, it should be possible to show whether the lower plant transit-peptide sequence will direct the NADP-GDH precursor-protein into the Arabidopsis chloroplast and will be recognized and processed by the endopeptidase(s) of this higher plant. Also, another question of comparative biochemistry importance is whether the natural promoter(s) of the Chlorella NADP-GDH gene will be recognized by the regulatory proteins/RNA polymerase of Arabidopsis. Moreover, if the NADP-GDH gene is transcribed into a large precursor mRNA (pre-mRNA) in this higher plant, will the many exons (including one only 18 bp) be spliced together correctly?

Efficiency of inorganic nitrogen assimilation, carbon dioxide fixation, and biomass yield in transgenic Arabidopsis plants expressing chloroplastic NADP-GDH

For every NH_4^+ assimilated into glutamate by the chloroplastic NADP-GDH rather than by the GS/GOGAT pathway, one ATP should be saved. A question of importance from an agricultural biotechnology standpoint is whether this savings in ATP can be translated into a net gain in energy that can be used for anabolic processes by the plant.

Before plant productivity studies are considered, several basic measurements need to be performed on the different (isolates) Arabidopsis transgenic plants having chloroplastic NADP-GDH activity. Due to variations in the number of copies (gene dosage) of the NADP-GDH cDNA/gene that can be inserted into the Arabidopsis genome and in their position(s) in the genome (i.e., adjacent genes/promoters can influence expression of inserted gene), different amounts of NADP-GDH activity may accumulate in the leaves. Firstly, it will be important to rank the transformants on the basis of their amount of leaf NADP-GDH activity. Their degree of resistance to chlorosis under photorespiratory conditions may prove to be correlated to the amount of NADP-GDH activity in their leaves. Secondly, the NH_4^+ concentration in the leaves will be measured before transfer to photorespiratory conditions and during a time-course thereafter. Thirdly, the photosynthetic rate will be measured (22) as a function of time after transfer to photorespiratory conditions. As controls, the same measurements will be performed on the wild-type and GS/GOGAT mutant (not transformed) Arabidopsis plants.

Because of the possible variation in gene dosage, there could be a wide range of NADP-GDH activities in the transformants. From a plant energy-economy standpoint, the ideal transgenic plants, selected for biomass production measurements, will be those with the lowest levels of NADP-GDH activity which can maintain wild-type levels (or lower) of NH_4^+ in the leaves under photorespiratory conditions. Because the NADP-GDH cDNA/gene insertions into the Arabidopsis genome might lower the activity of some essential plant enzyme unrelated to nitrogen metabolism, a number of NADP-

GDH transformants will be evaluated in the biomass productivity studies. The following comparisons will be made between wild-type Arabidopsis and the aforementioned final-selection of transformants during growth (in a random-block design) under photorespiratory conditions:

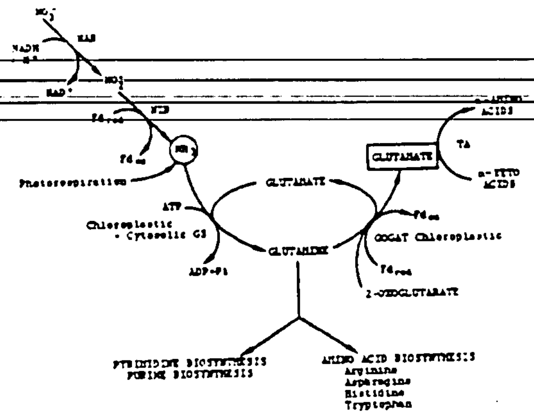
a. Generation time from seed germination until seed set.
b. ~~Total protein, total RNA, total DNA, lipid, starch, and chlorophyll content of leaves (per fresh and dry weight) at periodic intervals during growth/maturation cycle.~~
c. Total weight of seed produced.
d. Rates of uptake of NO_3^- and NH_4^+ in separate nutrition experiments vs. developmental stage.
e. Rate of $^{14}CO_2$ incorporation by the intact plant.
f. Leaf ADP/ATP ratio.

D. Figure Legend and Figure

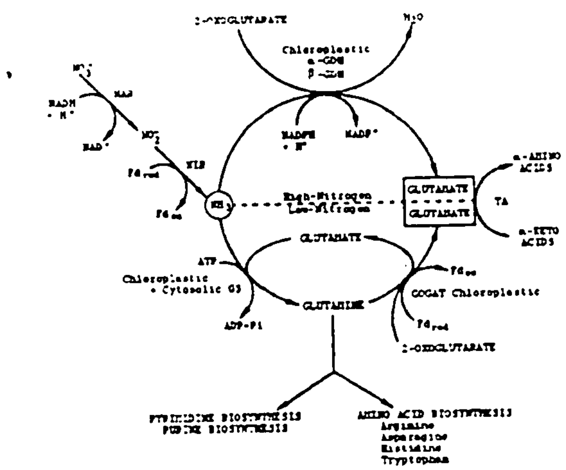
Fig. 1 A,B,C. Pathways of inorganic nitrogen assimilation/reassimilation in A. thaliana leaves, C. sorokiniana cells, and transgenic/mutant A. thaliana leaves.

FIGURE 1

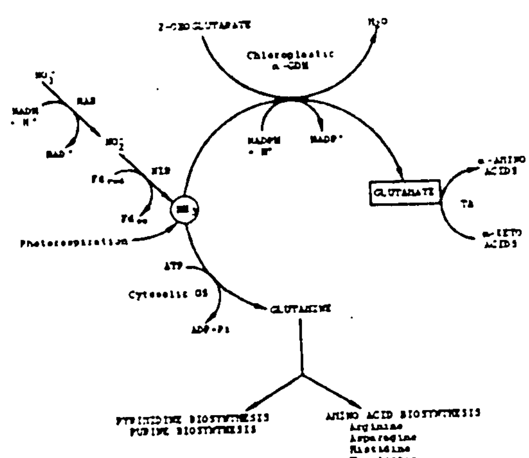
A. INORGANIC NITROGEN ASSIMILATION/REASSIMILATION IN WILD-TYPE ARABIDOPSIS THALIANA



B INORGANIC NITROGEN ASSIMILATION IN CHLORELLA SOROKINIANA



C. INORGANIC NITROGEN ASSIMILATION/REASSIMILATION IN TRANSGENIC MUTANT ARABIDOPSIS THALIANA



REFERENCES

1. Miflin, B.J., and P.J. Lea (1976) Photochemistry 15:873.
2. Miflin, B.J., and P.J. Lea (1980) In, The Biochemistry of Plants, B.J. Miflin (ed.), Academic Press, p. 169.
3. ~~Keys, A.L., Bird, I.F., Cornelius, M.F., Lea, P.L., Wallsgrove, R.M., and B.J. Miflin (1978) Nature 275:741.~~
4. Ogren, W.L. (1984) Annu. Rev. Plant Physiol. 35:415.
5. Givan, C.V., Joy, K.W., and L.A. Kleczkowski (1988) TIBS 13:433.
6. Wootton, J.C., and M.J. McPherson (1984) In, The Genetic Manipulation of Plants and Its Application to Agriculture, P.J. Lea and G.R. Stewart (eds.), Cavendon Press, p. 89.
7. Stewart, G.R., Mann, A.F., and P.A. Fentem (1980) In, The Biochemistry of Plants, Vol. 5, Academic Press, p. 271.
8. Meredith, M.J., Gronostajski, R.M., and R.R. Schmidt (1978) Plant Physiol. 61:967.
9. Schmidt, R.R., Turner, K.J., Bascomb, N.F., Thurston, C.F., Lynch, J.J., Molin, W.T., and A.T. Yeung (1982) In, Genetic Expression in The Cell Cycle, K.S. McCarty, Sr., and G.M. Padilla (eds.), Academic Press, p. 199.
10. Yeung, A.T., Turner, K.J., Bascomb, N.F., and R.R. Schmidt (1981) Anal. Biochem. 110:216.
11. Bascomb, N.F., and R.R. Schmidt (1987) Plant Physiol. 83:75.
12. Cock, J.M., Roof, L.L., Bascomb, N.F., Gehrke, C.W., Kuo, K.C., and R.R. Schmidt (1990) J. Phycol. 26, 361.
13. Schmidt, R.R. (1990) In, New Directions in Biological Control, UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 112, R. Baker and P. Dunn (eds.), Alan R. Liss, p. 1-22.
14. Cock, J.M., Kim, K.-D., Miller, P.W., Hutson, R.G. and R.R. Schmidt (1991) Sequence of a nuclear gene with many introns encoding chloroplastic NADP-specific glutamate dehydrogenases in *Chlorella sorokiniana*. Plant Mol. Biol., submitted 1991.
15. Cock, J.M., Kim, K.-D., Miller, P.W., and R.R. Schmidt (1991) Sequence and induction pattern of a mRNA encoding ammonium inducible NADP-specific glutamate dehydrogenase(s) in *Chlorella sorokiniana*. Plant Mol. Biol., submitted 1991.
16. Nadal-Ginard, B., Gallego, M.E., and A. Andreadis (1987) In, Genetic Engineering, Vol. 9, J.E. Setlow (ed), Plenum Press, p. 249.
17. Werneke, J.M., Chatfield, J.M., and W.L. Ogren (1989) The Plant Cell 1:815.
18. Perlman, D., Raney, R., and H.O. Halvorson (1984) Mol. Cell Biol. 4:1682.
19. Innis, M.A., Gelfand, D.H., Sninsky, J.J., and T.J. White (1990) PCR Protocols: A Guide to Methods and Applications, Academic Press, pp. 482.
20. Bascomb, N.F., Prunkard, D.E., and R.R. Schmidt (1987) Plant Physiol. 83:85.
21. Bascomb, N.F., and R.R. Schmidt (1991) ATP and calcium requirement for inactivation and degradation of chloroplastic NADP-specific glutamate dehydrogenase in permeabilized cells and cell-free extracts of *Chlorella sorokiniana*. Plant Physiol., in preparation.
22. Somerville, C.R., and W.L. Ogren (1980) Nature 286:257.
23. Wallsgrove, R.M., Turner, J.C., Hall, N.P., Kendall, A.C., and S.W.J. Bright (1987)

Plant Physiol. 83:155.
24. McNally, S.F., Hirel, B., Gadal, P., Mann, A.F., and G.R. Stewart (1983) Plant Physiol. 72:22.
25. Edwards, J.W., and Coruzzi, G.M. (1989) The Plant Cell 1:241.
26. Walker, E.L., and G.M. Coruzzi (1989) Plant Physiol. 91:702.
~~27. Lightfoot, D.A., Green, N.K., and J.V. Cullimore (1988) Plant Mol. Biol. 11:191.~~
~~28. Forde, B.G., Day, H.M., Turton, J.F., Wen-jun, S., Cullimore, J.V., and J.E. Oliver~~ (1989) The Plant Cell 1:391.
29. Estelle, M.A., and C.R. Somerville (1986) Trends in Genetics 2:89.
30. Bevan, M., and A. Goldsbrough (1988) In, Genetic Engineering, Vol. 10, J.K. Setlow (ed.), Plenum Press, p. 123.
31. Lloyd, A.M., Barnason, A.R., Rogers, S.G., Byrne, M.C., Fraley, R.T., and R.B. Horsch (1986) Science 234:464.
32. Feldman, K.A., and M.D. Marks (1987) Mol. Gen. Genet. 208:1.
33. Feldmann, K.A., Marks, M.D., Christianson, M.L., and R.S. Quatrano (1989) Science 243:1351.
34. Valvekens, D., vanMontagu, M., and M. vanLijsebettens (1988) Proc. Natl. Acad. Sci. USA 85:5536.
35. Bascomb, N.F., Turner, K.J., and R.R. Schmidt (1986) Plant Physiol. 81:527.
36. Prunkard, D.E., Bascomb, N.F., Robinson, R.W., and R.R. Schmidt (1986) Plant Physiol. 81:349.
37. Prunkard, D.E., Bascomb, N.F., Molin, W.T., and R.R. Schmidt (1986) Plant Physiol. 81:413.
38. Keegstra, K., and L.J. Olsen (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:471.
39. McPherson, M.J., and J.C. Wootton (1983) Nucleic Acids Res. 11:5257.
40. Kinnaird, J.H., and J.R.S. Fincham (1983) Gene 26:253.
41. Mattaj, I.W., McPherson, M.J., and J.C. Wootton (1982) FEBS Lett. 147:21.
42. Goldschmidt-Clermont, M., and M. Rahire (1986) J. Mol. Biol. 191:421.
43. Murray, E.E., Lotzer, J., and M. Eberle (1989) Nucleic Acids Res. 17:477.
44. Karlin-Neumann, G.A., Kohorn, B.D., Thornber, J.P., and E.M. Tobin (1985) J. Mol. Appl. Genet. 3:45.
45. Sharp, P.M., Cowe, E., Higgins, D.G., Shields, D.C., Wolfe, K.H., and F. Wright (1988) Nucleic Acids Res. 16:8207.

FACILITIES AND EQUIPMENT

Dr. Schmidt has a laboratory of 1,600 sq. ft. which has essentially all of the equipment required for modern research in plant and microbial biochemistry and molecular biology. Typical equipment items include: two large nucleotide sequencing ~~apparatus, 1 - Pharmacia Fast Protein Chromatograph with different types of analytical columns, multiple units for analytical and preparative slab-gel electrophoresis,~~ transilluminator with Polaroid camera, fraction collectors and monitors, density-gradient former and fractionators, 1 - ultracentrifuge, several refrigerated centrifuges, -70°C freezer, Gilford recording spectrophotometer, a laboratory personal computer connected to university VAX, etc. In addition, the department has scintillation counters, an oligonucleotide synthesizer, electron microscopes, etc. The university Interdisciplinary Center for Biotechnology Research (ICBR) has the protein sequencer, amino acid analyzers, LKB laser densitometer, DNA sequencer, DNA synthesizer, etc. The ICBR also has a core facility for isolation and production of monoclonal antibodies and also polyclonal antibodies.

Essential for this project is a large constant-temperature, fluorescent-lighted, sealed environmental chamber for culturing GS and GOGAT Arabidopsis mutant plants in a controlled atmosphere of 1% CO_2-air. We have successfully cultured Arabidopsis plants to maturity from seed in 4 to 6 weeks in this chamber. In addition, we have constructed 10 fluorescent-light shelves (3′ x 5′) for culturing wild-type or transgenic Arabidopsis plants in a constant temperature (22°C) culture room in normal air. All culturing of transgenic plants will be in the environmental chamber or culture room. No transgenic plants will be cultured outside of Dr. Schmidt's laboratory.

The culture room also has facilities for growing plant tissue cultures and also mass cultures of algae and bacteria, and it houses a Sharples continuous-flow centrifuge for harvesting large culture volumes. In addition, his laboratory has its own walk-in coldroom laboratory (104 sq. ft.), and a darkroom (55 sq. ft.) for development of autoradiograms and for viewing nucleic acids in gels with a transilluminator.

To facilitate the direction/advisement of his graduate students, Dr. Schmidt's office opens directly into his main laboratory where students have their laboratory benches and desks.

BIOGRAPHICAL SKETCH

Robert R. Schmidt, Ph.D.
Principal Investigator

Birthdate: February 18, 1933

Current Position: Graduate Research Professor

~~Education:~~

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Virginia Polytechnic Institute & State University	B.S.	1955	Plant and Microbiol. Sci.
University of Maryland	M.S.	1957	Plant Physiology
Virginia Polytechnic Institute & State University	Ph.D.	1961	Biochemistry

M.S. Degree Advisor: Dr. R.W. Krauss
Ph.D. Degree Advisor: Dr. K.W. King (Deceased)

Employment/Experience:
1961-64 Assistant Prof., Dept. Biochemistry & Nutrition, VPI&SU, Blacksburg, VA.
1964-67 Associate Prof., Dept. Biochemistry & Nutrition, VPI&SU, Blacksburg, VA
1967-80 Professor, Dept. Biochemistry & Nutrition, VPI&SU, Blacksburg, VA

Sept. 1976 - June 1977, Visiting Professor of Biological Sciences, in laboratory of Dr. R.T. Schimke, Department of Biological Science, Stanford University, Calif. I learned immunological and nucleic acid techniques used in measurements of specific enzyme synthesis and degradation; mRNA isolation and purification; cDNA synthesis and hybridization.
Dec. 1980 - June 1982, Professor and Chairman, Dept. Microbiology & Cell Science, University of Florida, Gainesville, FL.
July 1982 - present, Graduate Research Professor (i.e., highest rank that a Full Professor can hold at the Univ. of Florida) in same department.

Honors/Awards:
Certificate of Teaching Excellence (1978); American Society of Biological Chemists (1967) Sigma Xi Graduate Research Award (1961); Phi Kappa Phi (1955); Phi Sigma Undergraduate Research Award (1954); Bausch and Lomb Honorary Science Award (1951).

Graduate Research/Teaching Experience:
In the last 30 years, I have supervised graduate students in my laboratory to 35 advanced degrees; Supervised 13 postdoctoral research associates; 4 visiting professors, and 5 laboratory technicians. I have supported these personnel primarily on grants from NIH, NSF, USDA, and NASA.
I currently have a research group which consists of 4 graduate students, a senior

laboratory technician, and a Visiting Full Professor on a 12 month sabbatical leave.

For 13 years, I taught a 2-quarter advanced graduate-level course, entitled Genetic and Metabolic Control. In this course, regulation of gene expression in both procaryotes and eucaryotes was discussed in great detail with emphasis on current techniques and experimental approaches in molecular biology and nucleic acid biochemistry.

I currently teach a major section of the departmental graduate core course in the area of biochemistry and molecular biology of nitrogen assimilation.

Publications:

I currently have 51 regular research publications; 8 chapters in books, two technical comments, and several papers in preparation. Ten selected publications related to this project are listed below:

1. Cock, J.M., Kim, K.D., Miller, P.W., and Schmidt, R.R. (1991) Nucleotide sequence and ammonium induction pattern of the mRNA encoding chloroplastic NADP-specific glutamate dehydrogenase(s) in Chlorella sorokiniana. Plant Molec. Biol., submitted.

2. Cock, J.M., Kim, K.D., Miller, P.W., Hutson, R.G., and Schmidt, R.R. (1991) Sequence of a nuclear gene with many introns encoding chloroplastic NADP-specific glutamate dehydrogenases in Chlorella sorokiniana. Plant Molec. Biol., submitted.

3. Cock, J.M., Roof, L.L., Bascomb, N.F., Gehrke, C.W., Kuo, K.C., and Schmidt, R.R. (1990) Restriction enzyme analysis and cloning of high molecular weight genomic DNA isolated from Chlorella sorokiniana (Chlorophyta). J. Phycol. 26:361-367.

4. Schmidt, R.R. (1990) In, New Directions in Biological Control (UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 112; Baker, R., and Dunn, P., eds.), Alan R. Liss, New York. Investigation of mechanisms: the key to successful use of biotechnology, pp 1-22.

5. Cock, J.M., and Schmidt, R.R. (1989) A glutamate dehydrogenase gene sequence. Nucleic Acids Research 17:10500.

6. Bascomb, N.F., Prunkard, D.E., and Schmidt, R.R. (1987) Different rates of synthesis and degradation of two chloroplastic ammonium-inducible NADP-specific glutamate dehydrogenase isoenzymes during induction and deinduction in Chlorella sorokiniana cells. Plant Physiol. 83, 85-91.

7. Bascomb, N.F., and Schmidt, R.R. (1987) Purification and partial kinetic and physical characterization of two chloroplast-localized NADP-specific glutamate dehydrogenase isoenzymes and their preferential accumulation in Chlorella sorokiniana cells cultured at low or high ammonium levels. Plant Physiol. 83, 75-84.

8. Bascomb, N.F., Turner, K.J., and Schmidt, R.R. (1986) Specific polysome immunoadsorption to purify an ammonium-inducible glutamate dehydrogenase mRNA from Chlorella sorokiniana and synthesis of full length double-stranded cDNA from the purified mRNA. Plant Physiol. 81, 527-532.

9. Prunkard, D.E., Bascomb, N.F., Molin, W.T., and Schmidt, R.R. (1986) Effect of different carbon sources on the ammonium induction of different forms of NADP-specific glutamate dehydrogenase in Chlorella sorokiniana cells cultured in the light and dark. Plant Physiol. 81, 413-422.

10. Prunkard, D.E., Bascomb, N.F., Robinson, R.W. and Schmidt, R.R. (1986) Evidence ~~for chloroplastic localization of an ammonium-inducible glutamate dehydrogenase and synthesis of its subunit from a cytosolic precursor-protein in Chlorella~~ sorokiniana. Plant Physiol. 81, 349-355.

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BUDGET

FIRST YEAR

Organization and Address	USDA Grant No.	
University of Florida/Division of Sponsored Research 219 Grinter Hall Gainesville, FL 32611	Duration Proposed Months: 12	Duration Awarded Months: ______
Principal Investigator(s)/Project Director(s) Robert R. Schmidt, Graduate Research Professor	FUNDS REQUESTED BY PROPOSER	FUNDS APPROVED BY CSRS (If different)

	CSRS FUNDED WORK MONTHS: Calendar	Academic	Summer	FUNDS REQUESTED BY PROPOSER	FUNDS APPROVED BY CSRS (If different)
A. Salaries and Wages					
1. No. of Senior Personnel					
a. ______ (Co)-PI(s)/PD(s)				$ None	$
b. ______ Senior Associates					
2. No. of Other Personnel (Non-Faculty)					
a. ______ Research Associates-Postdoctorate					
b. ______ Other Professionals					
c. 3 Graduate Students (Ph.D. candidates)				33,000	
d. 2 Prebaccalaureate Students Part-time @ 4.75 hr				2,000	
e. ______ Secretarial-Clerical					
f. ______ Technical, Shop and Other					
Total Salaries and Wages				35,000	
B. Fringe Benefits (If charged as Direct Costs)					
C. **Total Salaries, Wages, and Fringe Benefits** (A plus B)				35,000	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)					
E. Materials and Supplies				20,000	
F. Travel 1. Domestic (Including Canada) National meetings				2,000	
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges				1,500	
H. Computer (ADPE) Costs					
I. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.) (Repair of major equipment items)				2,500	
J. **Total Direct Costs** (C through I)				61,000	
K. **Indirect Costs** (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.) 14% total direct costs				8,540	
L. **Total Direct and Indirect Costs** (J plus K)				69,540	
M. **Other**					
N. **Total Amount of This Request**				$69,540	$
O. **Cost Sharing**	$				

NOTE: Signatures required only for Revised Budget

This is Revision No. ➤

Name and Title (Type or print)	Signature	Date
Principal Investigator/Project Director		
Authorized Organizational Representative		

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COOPERATIVE STATE RESEARCH SERVICE

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BUDGET

SECOND YEAR

Organization and Address	USDA Grant No.	
University of Florida/Division of Sponsored Research 219 Grinter Hall Gainesville, FL 32611	Duration Proposed Months: 12	Duration Awarded Months:
Principal Investigator(s)/Project Director(s) Robert R. Schmidt, Gradaute Research Professor	FUNDS REQUESTED BY PROPOSER	FUNDS APPROVED BY CSRS (If different)

	CSRS FUNDED WORK MONTHS			FUNDS REQUESTED BY PROPOSER	FUNDS APPROVED BY CSRS (If different)
A. Salaries and Wages					
1. No. of Senior Personnel	Calendar	Academic	Summer		
a. ____ (Co)-PI(s)/PD(s)				$ None	$
b. ____ Senior Associates					
2. No. of Other Personnel (Non-Faculty)					
a. ____ Research Associates-Postdoctorate					
b. ____ Other Professionals					
c. 3 Graduate Students (Ph.D. candidates)				34,650	
d. 2 Prebaccalaureate Students (part-time @ 4.75 hr				2,000	
e. ____ Secretarial-Clerical					
f. ____ Technical, Shop and Other					
Total Salaries and Wages				36,650	
B. Fringe Benefits (If charged as Direct Costs)					
C. **Total Salaries, Wages, and Fringe Benefits** (A plus B)				36,650	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)					
E. Materials and Supplies				21,000	
F. Travel 1. Domestic (Including Canada) National meetings				2,000	
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges				1,500	
H. Computer (ADPE) Costs					
I. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.) (Repair of major equipment items)				2,500	
J. **Total Direct Costs** (C through I)				63,650	
K. **Indirect Costs** (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.) 14% total direct costs				8,911	
L. **Total Direct and Indirect Costs** (J plus K)				72,561	
M. **Other**					
N. **Total Amount of This Request**				$72,561	$
O. **Cost Sharing**	$				

NOTE: Signatures required only for Revised Budget

This is Revision No. ➤

Name and Title (Type or print)	Signature	Date
Principal Investigator/Project Director		
Authorized Organizational Representative		

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THIRD YEAR

Organization and Address	USDA Grant No.	
University of Florida/Division of Sponsored Research 219 Grinter Hall Gainesville, Fl 32611	Duration Proposed Months: 12	Duration Awarded Months:
Principal Investigator(s) Project Director(s) Robert R. Schmidt, Graduate Research Professor	FUNDS REQUESTED BY PROPOSER	FUNDS APPROVED BY CSRS (If different)

	CSRS FUNDED WORK MONTHS			FUNDS REQUESTED BY PROPOSER	FUNDS APPROVED BY CSRS (If different)
A. Salaries and Wages	Calendar	Academic	Summer		
1. No. of Senior Personnel					
a. ____ (Co)-PI(s)/PD(s)				$ None	$
b. ____ Senior Associates					
2. No. of Other Personnel (Non-Faculty)					
a. ____ Research Associates-Postdoctorate					
b. ____ Other Professionals					
c. 3 Graduate Students (Ph.D. candidates)				36,383	
d. 2 Prebaccalaureate Students (part time @ 4.75 hr)				2,000	
e. ____ Secretarial-Clerical					
f. ____ Technical, Shop and Other					
Total Salaries and Wages ➤				38,383	
B. Fringe Benefits (If charged as Direct Costs)					
C. Total Salaries, Wages, and Fringe Benefits (A plus B) ➤				38,383	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)					
E. Materials and Supplies				22,050	
F. Travel 1. Domestic (Including Canada) National meetings				2,000	
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges				1,500	
H. Computer (ADPE) Costs					
I. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.) (Repair of major equipment items)				2,500	
J. Total Direct Costs (C through I) ➤				66,433	
K. Indirect Costs (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.)				9,301	
L. Total Direct and Indirect Costs (J plus K) ➤				75,734	
M. Other ➤					
N. Total Amount of This Request ➤				$ 75,734	$
O. Cost Sharing	$				

NOTE: Signatures required only for Revised Budget

This is Revision No. ➤

Name and Title (Type or print)	Signature	Date
Principal Investigator/Project Director		
Authorized Organizational Representative		

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BUDGET

CUMULATIVE SUMMARY

Organization and Address	USDA Grant No.	
University of Florida/Division of Sponsored Research 219 Grinter Hall Gainesville, FL 32611	Duration Proposed Months: 36 FUNDS REQUESTED BY PROPOSER	Duration Awarded Months: ____ FUNDS APPROVED BY CSRS (If different)
Principal Investigator(s)/Project Director(s) Robert R. Schmidt, Graduate Research Professor		

	CSRS FUNDED WORK MONTHS: Calendar	Academic	Summer	FUNDS REQUESTED BY PROPOSER	FUNDS APPROVED BY CSRS (If different)
~~A. Salaries and Wages~~					
~~1. No. of Senior Personnel~~					
a. ____ (Co)-PI(s)/PD(s)				$ None	$
b. ____ Senior Associates					
2. No. of Other Personnel (Non-Faculty)					
a. ____ Research Associates-Postdoctorate					
b. ____ Other Professionals					
c. 3 Graduate Students (Ph.D. candidates)				104,033	
d. 2 Prebaccalaureate Students (part-time @ 4.75 hr)				6,000	
e. ____ Secretarial-Clerical					
f. ____ Technical, Shop and Other					
Total Salaries and Wages ➤				110,033	
B. Fringe Benefits (If charged as Direct Costs)					
C. **Total Salaries, Wages, and Fringe Benefits** (A plus B) ➤				110,033	
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)					
E. Materials and Supplies				63,050	
F. Travel 1. Domestic (Including Canada) National meetings				6,000	
2. Foreign (List destination and amount for each trip.)					
G. Publication Costs/Page Charges				4,500	
H. Computer (ADPE) Costs					
I. All Other Direct Costs (Attach supporting data. List items and dollar amounts. Details of subcontracts, including work statements and budget, should be explained in full in proposal.) (Repair of major equipment items)				7,500	
J. **Total Direct Costs** (C through I) ➤				191,083	
K. **Indirect Costs** (Specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs included in on/off campus bases.) 14% total direct costs				26,752	
L. **Total Direct and Indirect Costs** (J plus K) ➤				217,835	
M. **Other** ➤					
N. **Total Amount of This Request** ➤				$217,835	$
O. **Cost Sharing**	$				

NOTE: Signatures required only for Revised Budget

This is Revision No. ➤

Name and Title (Type or print)	Signature	Date
Principal Investigator/Project Director		
Authorized Organizational Representative		

Form CSRS-55 (9/89)

BUDGET JUSTIFICATION

Personnel:

1. Principal Investigator:
   Dr. Schmidt will spend 25% of his time on this project. No salary funds are requested.

2. Three Graduate Research Assistants:
   Mr. Richard Hutson received a B.S. degree in Microbiology from the Virginia Polytechnic Institute and State University, and will receive the M.S. degree in molecular biology under my direction in He will pursue his Ph.D. in my laboratory.

   Mr. Philip Miller received a M.S. degree in Genetics from Appalachian State University and joined my laboratory Spring Semester 1990 and is pursuing his Ph.D. in molecular biology under my direction.

   Ms. Brenda Russell received a M.S. degree in Microbiology from the Virginia Polytechnic Institute & State University and joined my laboratory Summer Semester 1990 and is pursuing her Ph.D. in molecular biology under my direction.

   Each of these graduate students is currently working on aspects of the molecular biology of the Chlorella and Arabidopsis project. Their continued work in this area requires an extramural grant.

3. Laboratory Aids:
   Part-time undergraduate-student employees are required to wash and/or sterilize the large volume of dirty laboratory glassware and culture tubes, etc. generated by an active research group. These part-time laboratory aids also are involved in the general laboratory maintenance required in a biochemistry/molecular biology laboratory.

4. Laboratory Technician (State funded):
   Ms. Waltraud Dunn, a senior level state-funded laboratory technician will devote approximately 25% of her time to this project with no funds requested for her salary from the NSF.

The salaries of the graduate students will be increased by 5% each year. There is a Graduate Student Union at the University of Florida that negotiates raises each year which range between 4% and 6%.

Travel:

Funds are requested to give talks/posters at the national meetings of the American Society of Biological Chemists, American Society of Plant Physiologists, and the American Society of Microbiology. The principal investigator, and graduate students will be attendees provided talks/posters are presented.

Materials and Supplies (per year):

1. Radioactive compounds, enzyme substrates, protein standards, restriction enzymes and other recombinant DNA reagents and linkers, translation assay components, Protein A, and other biochemical reagents, etc. $11,000

2. ~~Chromatography, electrophoresis, chromatofocusing columns, gels, packings, affinity resins, cellulose nitrate paper and other derivatized papers, polybuffers, etc. $3,000~~

3. Glassware, plasticware, scintillation vials, Eppendorf pipettes tips, distilled H_2O dionizer cartridges, culture tubes, microcentrifuge tubes, liquid nitrogen, carbon dioxide, argon, X-ray film, etc. $3,000

4. Small equipment items costing less than $500 will be required for this project, e.g., automatic pipettes, special electrophoresis chambers, dialysis chambers, pumps, thermoregulators, magnetic stirrers, heaters, etc. $3,000

   Because of the rapid increase in costs of biochemical and molecular biology reagents, a 5% increase per year is budgeted.

Publication Costs/Page Charges:

The funds are requested for page costs and also for making photographs of gels, autoradiograms, etc. and for preparation of figures for publication. With research progressing so rapidly, it is anticipated that equal funding will be required each year for publication related costs.

Other Direct Costs:

The costs for repairs of power supplies, centrifuges, low temperature freezers, Coulter cell counter, spectrophotometer, freezer drier, fraction collectors, etc. routinely costs a minimum of $2,500 per year.

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UNITED STATES DEPARTMENT OF AGRICULTURE
COOPERATIVE STATE RESEARCH SERVICE

ASSURANCE STATEMENT(S)

STATEMENT OF POLICY - Safeguarding the rights and welfare of subjects at risk and the proper isolation security of research agents in activities supported by Cooperative State Research Service is the responsibility of the institution to which support is provided. In order to provide for the adequate discharge of this responsibility, USDA policy requires a formal assurance that appropriate committees in each institution will carry out both initial review of proposals and continuing review of supported projects. The Department also requires certification of such reviews.

NOTE: Check appropriate statements, supplying additional information when necessary

1. Institution University of Florida	2. Type New Extension Revision 3. Project Number or Grant Number (If Known)
4. Title of Project Development of Transgenic C_3 Plants Requiring Less ATP for NH_4^+ Assimilation	5. Principal Investigator(s) Robert R. Schmidt

A. RECOMBINANT DNA OR RNA RESEARCH

This institution agrees to assume primary responsibility for complying with both the intent and procedures of the National Institutes of Health's (NIH) "Guidelines for Research Involving Recombinant DNA Molecules," as revised (see subsection 205(b)(3), Subpart U of the "Uniform Federal Assistance Regulations" (7 CFR Part 3015)) and other applicable Federal/State guidelines and regulations.

This responsibility includes:

1. Ensuring that a standing Institutional Biosafety Committee (IBC) reviews proposed projects.
2. Registering with the IBC all experiments involving recombinant DNA and RNA Molecules conducted with the funds provided under this project/grant and complying with the requirements specified in Part II of the NIH Guidelines or any other pertinent guidelines and regulations. IBC's are required to keep records of this research in a form that is available to the U.S. Department of Agriculture (USDA) upon request.

In addition, principal investigators must report the following to the USDA and to their IBC's:

1. New technical information relating to risks and safety procedures.
2. Serious accidents or releases involving recombinant DNA or RNA.
3. Serious illness of a laboratory worker which may be project related.
4. Other safety problems.

☐ **Project does not involve recombinant DNA or RNA.**

X **Project involves recombinant DNA or RNA.** (Check applicable statement(s)).

☐ This project has been determined by the local IBC to be exempt from the NIH Guidelines.

XX This project has been reviewed by an IBC and was approved on 11-23-83 and revised 7/3/86 (Date). for project with Chlorella.

XX Other action (explain) The revised DOR for extending recombinant DNA research to include the insertion of Chlorella NADP-GDH gene into Arabidopsis is currently being prepared for submission to the local IBC.

B. ANIMAL CARE

XX **Project does not involve use of vertebrate animals.**

☐ **Project involves use of vertebrate animals.** *(Check the following applicable statement(s)).*

☐ a) This project is in compliance with the Animal WelfareAct of 1966 and 9 CFR Subchapter A (Laboratory Animals), as amended.

☐ b) This project is under review by the Institutional Care and Use Committee and a revised Form CSRS-662 will be submitted when the review is completed.

☐ c) This project has been approved by the Institutional Animal Care and Use Committee on ______________(Date).

Signature of Authorized Organizational Representative	Title	Date

(OVER)

Form CSRS-662 (9/89) Previous editions of this form are obsolete.

APPENDIX

Two manuscripts submitted March 1991:

1. Cock, J.M., Kim, K.D., Miller, P.W., and Schmidt, R.R. (1991) Sequence and ammonium induction pattern of a mRNA encoding chloroplastic NADP-specific ~~glutamate dehydrogenase(s) in Chlorella sorokiniana. Plant Molec. Biol., submitted.~~

2. Cock, J.M., Kim, K.D., Miller, P.W., Hutson, R.G., and Schmidt, R.R. (1991) Sequence of a nuclear gene with many introns encoding chloroplastic NADP-specific glutamate dehydrogenases in Chlorella sorokiniana. Plant Molec. Biol., submitted.

#1

Sequence and Ammonium Induction Pattern of a mRNA Encoding Chloroplastic NADP-Specific Glutamate Dehydrogenase(s) in *Chlorella sorokiniana*

J. Mark Cock,[1] Kyu Don Kim, Philip W. Miller, and Robert R. Schmidt*

Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611;[1] *Present address: Laboratoire de Biologie Moléculaire des Relations Plantes - Micro-organismes, INRA-CNRS, 31326 Castanet-Tolosan Cédex, France* (*author for correspondence)

Running Title: Glutamate dehydrogenase mRNA sequence/regulation.

Accession number: ________

Key words: ammonium inducible, cDNA library/sequences, *Chlorella sorokiniana*, gene expression, glutamate dehydrogenase, mRNA synthesis/degradation

Abstract

A cDNA library was prepared from total poly(A)$^+$RNA extracted from *Chlorella sorokiniana* cells which were simultaneously synthesizing two ammonium-inducible chloroplastic NADP-specific glutamate dehydrogenase (NADP-GDH) isoenzymes. These isoenzymes have strikingly different affinities for ammonium and are composed of α- and β-subunits of different sizes but with very similar peptide maps. Seventeen independent NADP-GDH cDNA clones were isolated. Comparison of their restriction maps and nucleotide sequences suggests that all of them were derived from a single mRNA species. Each cDNA contained a highly conserved region, with a deduced amino acid sequence, having 77% and 73% identity with the corresponding regions in the NADP-GDH genes of *Escherichia coli* and *Neurospora crassa*. The longest cDNA was fused in frame with *lac* z in a Bluescript vector, and was expressed as NADP-GDH antigen in *E. coli*. From the 17 cDNAs, a 2,145 bp consensus sequence was derived which encodes a 57,401 Da protein of nearly the same size (58,500 Da) as the precursor-protein(s) from which the subunits are processed. During a 240 min induction period, under conditions in which both types of subunits were synthesized, only a single-size (2.2 kb) NADP-GDH mRNA was detected using cDNA probes corresponding to the highly conserved region or 3′-untranslated region of the consensus cDNA. These results are consistent with a single mRNA encoding a precursor-protein which is differentially processed to yield α- and β-subunits.

Introduction

In this laboratory, *Chlorella sorokiniana* cells have been shown [3,26] to synthesize three ~~different glutamate dehydrogenase (GDH) isoenzymes: a constitutive, mitochondrial, tetrameric (subunit M_r = 45,000) NAD-specific isoenzyme (NAD-GDH), and two~~ ammonium-inducible, chloroplastic, hexameric NADP-specific (NADP-GDH) α- and β-isoenzymes (subunits M_r = 55,500 and 53,000, respectively). Only the α-homohexamer accumulates in the chloroplast of cells cultured in medium containing 1-2 mM ammonium [3]. The addition of higher ammonium concentrations (3.4 - 29 mM) to uninduced cells results in accumulation of both types of subunits (α and β) in NADP-GDH holoenzymes for the first 120 min. Thereafter, only the β-homohexamer accumulates [2,3,32]. When the α- and β-subunits are concomitantly synthesized early in the induction period in 29 mM ammonium medium, Prunkard *et al.* [32] detected seven different electrophoretic-forms of the NADP-GDH holoenzymes during native-PAGE. These forms were shown to have different molecular weights, presumably resulting from the formation of homo- and heterohexamers due to random-mixing of α- and β-subunits (i.e., 6α, $5\alpha{:}1\beta$, $4\alpha{:}2\beta$, $3\alpha{:}3\beta$, $2\alpha{:}4\beta$, $1\alpha{:}5\beta$, 6β).

The purified α- and β-homohexamers have strikingly different ammonium K_m values [3]. However, the K_m values for their other substrates are very similar. The α-homohexamer is an allosteric enzyme in that its ammonium K_m ranges from 0.02 to 3.5 mM, depending upon the NADPH concentration. In contrast, the β-homohexamer is non-allosteric and has an ammonium K_m of approximately 75 mM. None of the heterohexamers have been purified from each other to determine how their ammonium K_m values differ from those of the two homohexamers. When homo- and heterohexamers of NADP-GDH were accumulating during early induction in 29 mM ammonium, Bascomb *et al.* [2] performed pulse-chase experiments and determined that the α-subunit antigen was degraded with a half-life of 50 min whereas the β-subunit antigen was degraded more slowly with a half-life of 150 min. After the removal of ammonium from these induced cells, enhanced rates of degradation were observed for the α- and β-subunit antigens, i.e., half-lifes of 5 min and 13.5 min, respectively.

Although the α- and β-homohexamers have different affinities for ammonium and their rates of *in vivo* turnover are very different, they were shown [3,33] to have very similar peptide maps and to be derived from precursor proteins of identical size (M_r = 58,500). Moreover, antibodies prepared against one of the homohexamers will immunoprecipitate both isoenzymes [3,45]. These latter biochemical and immunochemical properties indicate that the α- and β-subunits have a considerable amount of sequence homology. We were therefore prompted to consider whether these two subunits might arise from (i) the differential processing of a precursor-protein encoded by a single mRNA and gene, (ii) the specific processing of two very similar precursor-proteins encoded by two mRNAs formed by alternative splicing [30,42] of a precursor mRNA transcribed from a single gene, or (iii) the specific processing of two precursor-proteins encoded by two mRNAs transcribed from two closely related genes. The purpose of the research described in this paper was to determine how many mRNAs encode the α- and β-subunits. Our experimental findings are consistent with but do not prove that a single NADP-GDH mRNA encodes a precursor-protein which is processed to give either α- or β-subunits.

COCK

In the following paper [7], the sequence of the nuclear gene encoding this NADP-GDH mRNA is presented.

Materials and methods

Preparation and screening of a C. sorokiniana *cDNA library*

~~Uninduced *C. sorokiniana* cells were synchronized by intermittent illumination (9h light:7h dark) in 29 mM nitrate medium [32] as previously described [40], except that the~~ EDTA concentration in the medium was increased from 0.072 mM to 0.31 mM to prevent precipitation of salts during autoclaving. The light intensity was adjusted to support a growth rate at which each cell would divide into four progeny at the end of each cell cycle.

Uninduced synchronized daughter cells were washed in nitrogen-free culture medium, and then transferred into medium containing 29 mM ammonium, and the culture was immediately placed into the light in a 3 L Plexiglas chamber to induce the accumulation of the NADP-GDH α- and β-isoenzymes. Samples were taken every 20 min to monitor induction of NADP-GDH activity [40], culture pH, and culture turbidity (A_{640}). The culture was harvested after 80 min of induction. At this induction time, the cells were still accumulating both NADP-GDH isoenzymes, and the α- and β-subunits each constituted about 50% of the total NADP-GDH antigen [2,32].

The cells were frozen (-70°C) as a suspension of 1 g fresh weight of cells per 5 ml in 0.1 M Tris-HCl pH 8.2. Two grams of cells were washed once in 0.1 M Tris-HCl (pH 8.5), and resuspended in 50% (w/v) guanidine thiocyanate, 0.5% (w/v) sodium lauryl sarcosine, 25 mM EDTA, 0.5% (w/v) antifoam A. The cells were broken by two passages through a 5 ml French pressure cell at 20,000 p.s.i. (i.e., 138 MPa). Total cellular RNA was purified by centrifugation to a pellet through a cushion of 5.7 M CsCl overlayed with 2.4 M CsCl, and by extraction with phenol as described by Maniatis *et al.* [22]. Total poly(A)$^+$RNA was isolated by oligo(dT) chromatography as described by Turner *et al.* [39]. As a test of mRNA integrity, samples of this poly(A)$^+$RNA preparation were shown to support a high level of protein synthesis in a mRNA-dependent rabbit reticulocyte lysate *in vitro* translation system [31,39].

The purified poly(A)$^+$RNA was sent to Stratagene Cloning Systems (Palo Alto, CA) where a cDNA library was constructed by the addition of *Eco*RI linkers to double-stranded cDNA followed by ligation into λgt10 [17]. The primary library contained 5 x 10^6 clones, and these primary clones were amplified to 2 x 10^{10} pfu per ml in *E. coli* strain C600 Hfl$^-$. In the initial screening of the amplified library, the DNA in 1 x 10^6 phage plaques was adsorbed to nylon membrane filters (Hybond N, Amersham). A radioactive (2.75 x 10^9 cpm/1.5 μg) heterologous NADP-GDH DNA probe was synthesized [12,13] from random hexamer primers (Pharmacia) annealed to 1.5 μg of the 1.2 kb *Hind*III/*Eco*RI fragment of pJB103 [27], and this probe was used for the three rounds of screening. The clone pJB103 was kindly provided as a gift by Drs. J.E. Brenchley and E.S. Miller (Penn State University and North Carolina State University, respectively).

Lambda phage containing hybridizing cDNA inserts were isolated using an immunoadsorption procedure (LambdaSorb; Promega). DNA was extracted from these phage and the cDNA insets were released by *Eco*RI digestion, subcloned into pUC18 [44], and transformed into *E. coli* DH5α [16]. Putative NADP-GDH cDNA clones in

pUC18 were designated as the pGDc series. The cDNA inserts were analyzed by restriction mapping and nucleotide sequencing.

After mapping and sequencing the first set of NADP-GDH cDNAs, the amplified ~~library was screened once again but with a homologous 115 bp probe derived from near the 5′ terminus but including part of the highly conserved region of the pGDc23~~ sequence. The putative NADP-GDH cDNAs, isolated in this second screening, were also mapped and partially sequenced.

Sequencing of NADP-GDH cDNA clones

cDNA clone pGDc23 was completely sequenced in both directions and the other NADP-GDH cDNAs were partially sequenced from both ends. This procedure involved subcloning *Eco*RI, *Pst*I, and *Eco*RI/*Bgl*II restriction fragments (from the cDNA inserts in pUC18) into M13 mp 18/19 [44] and also generation of a set of nested deletions from each end of the insert by digestion with exonuclease *Bal*31 [22] also followed by subcloning into M13 mp 19. Fragments cloned into M13 were sequenced by the dideoxy method of Sanger *et al.* [35] using a modified bacteriophage T7 polymerase [38] (Sequenase, United States Biochemical Corp.). The DNA sequence data were analyzed by use of Pustell sequence analysis programs (International Biotechnologies, Inc.)

Expression of pGDc23 cDNA in E. coli

E. coli strain JM109 [44] was transformed with pBluescript SK$^-$ (Stratagene Cloning Systems, Inc.) carrying pGDc23 fused in frame to a portion of the *lac* z gene and under control of the lac promoter. This construct was designated pBS-GDc23. The bacterial cells were cultured at 37°C in 2 x TY medium to a culture turbidity (A_{600}) of 0.70, IPTG was added to 5 mM, and the cells were induced for 3 h. For preparation of a crude cell extract, the cells (4.8 A_{600} units/ml) were pelleted at 1,500 x *g* for 10 min, resuspended in 0.35 ml of 55 mM Tris-HCl pH 6.8, 2.8 M β-mercaptoethanol, 0.0025% bromophenol blue, 4% SDS, 7.7% glycerol, boiled for 5 min, and centrifuged for 5 min at 15,000 x *g* in an Eppendorf microfuge. One tenth of this extract was subjected to SDS denaturing electrophoresis in a 10% polyacrylamide gel (16 h at 50 v). The proteins were electrophoretically transferred from the gel to a nitrocellulose membrane and the NADP-GDH antigen was detected by an immunochemical procedure [32] using ^{125}I-labelled Protein A and anti-NADP-GDH IgG [45]. The dried membrane was exposed to Kodak X-Omat AR film for 36 h at -70°C.

Kinetics of ammonium induction of NADP-GDH mRNA and catalytic activity

Synchronized *C. sorokiniana* cells were induced in 29 mM ammonium medium as described above. After addition of ammonium to the cells, samples of 500 ml of cell suspension (approximately 140 x 10^6 cells/ml) were harvested at zero time and at 20 min intervals for the first 140 min and a final sample was harvested at 240 min. The cells were then harvested by centrifugation at 10,000 x *g* for 5 min at 4°C, and fresh weight of the cells was measured. The cells were resuspended in 4 ml of 0.1 M Tris-HCl pH 8.5

per g of cells and frozen at -80°C. Total cellular RNA was extracted from 2 g pellets of cells as previously described [3,33]. Poly(A)$^+$RNA was isolated on an oligo(dT) column as described by Turner *et al.* [39]. The quantitation of poly(A)$^+$RNA in the various RNA ~~preparations was based on the formation of ribonuclease-resistant hybrids with ^{3}H-labelled polyuridylate (poly(^{3}H-5,6-uridylate), 2-10 Ci/mmol, New England Nuclear) as described earlier [5,10] using purified β-globin mRNA (Bethesda Research Laboratories)~~ as a standard. The yield of poly(A)$^+$RNA was between 37 and 90 μg per g of cells harvested during the induction periods. The NADP-GDH induction experiment was repeated to verify the mRNA induction pattern.

NADP-GDH catalytic activity was measured in the deaminating direction by the spectrophotometric procedure described by Turner *et al.* [40]. One unit of activity was defined as the amount of enzyme required to reduce 1 μmol of coenzyme/min at 38.5°C.

Northern blot analysis

RNA was electrophoresed in 2% agarose gels in the presence of 6% formaldehyde and blotted onto nylon membranes (Hybond N, Amersham) [9,22]. The RNA was crosslinked to the membrane by illumination with UV light for 5 min. The 4.5 h prehybridization and 16 h hybridization were described previously [9]. The radioactive probes were synthesized by use of random hexamer primers as described above. After hybridization, the membranes were washed three-times for 5 min with 2 x SSC, 0.1% SDS at room temperature (22°C) and then with 0.3 x SSC, 0.1% SDS for 5 min at room temperature. Autoradiography was performed as described above. The intensities of the NADP-GDH mRNA bands on the autoradiogram were quantified by a SCR ID/2D soft laser scanning densitometer (Biomed Instruments, Inc.).

Results and discussion

Isolation, restriction mapping, and sequencing of C. sorokiniana *NADP-GDH cDNAs*

~~*Bascomb et al.* [2] used a Western immmunoblotting procedure to show that α- and β-~~ subunits rapidly accumulate as NADP-GDH holoenzymes between 40 and 120 min after addition of 29 mM ammonium to previously uninduced *C. sorokiniana* cells. Free NADP-GDH subunits could not be detected in cell extracts. By pulse-chase studies, they determined that the α-subunit was synthesized five-times faster than the β-subunit and that both subunits underwent rapid turnover (i.e., degradation) during the aforementioned induction time-period. These findings led us to consider that, if the α- and β-subunits are encoded by different mRNAs, both mRNAs should be present between 40 and 120 min to sustain the linear accumulations of these subunits in view of their rapid and continuous turnover. However, based upon the faster rate of synthesis of the α-subunit, its mRNA would be predicted to be more abundant than the β-subunit mRNA. To determine how many mRNAs encode the α- and β-subunits, our experimental strategy was to prepare a cDNA library (from total poly(A)$^+$RNA extracted from cells induced in 29 mM ammonium for 80 min), isolate and sequence 15-20 NADP-GDH cDNAs from this library, and search for sequence differences among these cDNA clones.

The bacterial genes encoding NADP-GDH have been designed *gdh*A [25,27,28,41]. These genes have been shown to have a high degree of sequence homology with the *am* gene which encodes the NADP-GDH in the eukaryotic microorganism, *Neurospora crassa* [21]. The highest sequence homology is seen in a 354 bp region [24] near the 5′ termini of these genes, corresponding to a region near the amino-terminus of the NADP-GDHs. Because of this sequence homology between the conserved regions of the bacterial and eukaryotic NADP-GDHs, we decided to use this region from the cloned *S. typhimurium gdh*A gene [27] as an initial hybridization probe to screen the amplified *Chlorella* cDNA library in λgt10. The probe was a 1.2 kb *Eco*RI/*Hind*III restriction fragment from this gene which contained the highly conserved region near the 5′ end of the coding sequence.

Approximately 2 x 10^6 phage plaques were screened with the heterologous probe at low stringency and six putative NADP-GDH cDNAs were isolated. These cDNAs ranged in size from 0.6 to 1.91 kb, and their restriction maps/nucleotide sequences were found to be identical for their regions which overlapped (Fig. 1B). They appeared to be truncated forms of the longest cDNA which was designated pGDc23. In an attempt to select additional NADP-GDH cDNAs with longer 5′-termini than pGDc23, the cDNA library was rescreened at low stringency with a 115 bp *Pst*I restriction fragment (Fig. 1C) excised from near the 5′-terminus of pGDc23. This 115 bp homologous proximal-probe was chosen because approximately one-half of its sequence contains the 5′-terminus of the 354 bp conserved region (Fig. 1A) which would be predicted to be present in other types of NADP-GDH cDNAs if they exist. By use of this homologous probe, 11 additional NADP-GDH cDNAs were isolated, restriction mapped, and their 3′- and 5′-termini sequenced (Fig. 1C). These additional cDNAs also had identical restriction maps/sequences for the regions that overlapped. Although three of these cDNAs (i.e.,

pGDc31, 38, and 42) were longer than pGDc23 at their 5′-termini, none of them were full length.

Even though the cDNA library was prepared using an oligo(dT)primer, 10 of the 17 NADP-GDH cDNAs lacked a poly(A) tail and additional 3′-terminal sequences (Fig. ~~1B,C). Truncation at the 3′-terminus presumably resulted from secondary structure which formed and interfered with second-strand cDNA synthesis by pol I following first-~~ strand synthesis by reverse transcriptase. Moreover, because of premature termination of the reverse transcriptase during first-strand synthesis, none of the 17 cDNAs were full length at their 5′-termini. From the combined sequences of the 17 cDNAs, a 2,145 bp consensus NADP-GDH cDNA restriction map was constructed (Fig. 1A). Because Bascomb *et al.* [4] showed earlier that a 2.2-2.3 cDNA could be synthesized from highly purified *C. sorokiniana* NADP-GDH mRNA, isolated by a very specific polysome immunoselection procedure, full length NADP-GDH cDNA was anticipated to be at least 2.2 kb. As described in a later section of this paper, Northern blotting analyses showed the NADP-GDH mRNA to be 2.2 kb.

Analysis of the C. sorokiniana *NADP-GDH cDNA sequence*

A truncated open-reading-frame (ORF) was found running from the first nucleotide of the consensus NADP-GDH sequence to a TAA stop-codon at 1,570 bp (Fig. 2). This ORF encodes a protein with a molecular weight of 57,401 which is almost the complete size (~98%) of the NADP-GDH precursor-protein(s) (58,500 Da). The molecular weight of the mature α-subunit is 55,500; therefore, the consensus cDNA appears to encode part of the chloroplast transit-peptide [20] but is missing the ATG start-codon for this peptide and also the 5′-untranslated region of the mRNA. If the α- and β-subunits are formed by differential processing of a common precursor-protein derived from a single mRNA, this consensus cDNA presumably contains the sequences for both mature subunits.

The deduced amino acid sequence of the *C. sorokiniana* NADP-GDH is 50% and 50.3% identical with those of *E. coli* [25] and *N. crassa* [21], respectively. However, comparison of the sequences of the highly conserved region identified by Mattaji *et al.* [24] shows much stronger homologies of 76.6% and 73.4%, respectively (Fig. 2). The conserved region has been proposed to include amino acids involved in binding of dicarboxylate substrates, in catalytic activity, and in interactions affecting an allosteric conformational equilibrium [24]. The less conserved C-terminal halves of GDHs are probably involved in binding of their coenzymes [1,43].

Analysis of codon usage in the NADP-GDH encoding ORF (Table 1) revealed a strong bias towards the use of codons containing G and C at both the first (i.e., in the case of arginine and leucine codons) and the third positions. This preference correlated with the high GC content (63%) reported for *C. sorokiniana* genomic DNA [8]. Furthermore, for most amino acids, there was an extreme preference for a particular base at the third position of the codon (i.e., G for leucine and valine codons and C for serine, proline, threonine, arginine and glycine codons).

Expressed ribulose bisphosphate carboxylase small subunit genes of *Chlamydomonas reinhardtii* [15] exhibit the same extreme preference for the codons most frequently used

in the *Chlorella* NADP-GDH gene. This bias may prove to be a common feature for genes of eukaryotic algae. Genes of monocotyledonous plants also show a preference for codons containing G or C as the third degenerate base [29], especially in highly ~~expressed genes such as the chlorophyll a/b binding protein gene of *Lemna gibba* [19]. The codons utilized most frequently by the NADP-GDH gene also occur at a higher frequency in genes from *Drosophila* and GC rich mammalian genes but are not used~~ preferentially in genes of *E. coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [37].

Expression of C. sorokiniana *NADP-GDH antigen in* E. coli.

A translational fusion of the 5′ end of a modified *E. coli lac* gene with the long ORF of *C. sorokiniana* NADP-GDH cDNA clone pGDc23 was created by subcloning the cDNA insert into pBluescript SK$^-$ (pBS-GDc23; Fig. 3). *E. coli* strain JM109, carrying this construct, accumulated antigen which was recognized by antibodies raised to purified *C. sorokiniana* NADP-GDH (Fig. 3). A low level of transcription was initiated at the *lac* promoter in the absence of inducer, and the β-galactosidase-GDH fusion protein could be detected under these conditions (Fig. 3, lane 2). The addition of 5 mM IPTG resulted in approximately two-fold increase in the concentration of the fusion protein in the cell (Fig. 3, lane 3). The cDNA insert in pBS-GDc23 is 87% of the full length NADP-GDH mRNA (and encodes a protein of 440 amino acids with a molecular weight of 51,059). Therefore, this truncated subunit along with its 39 amino acid extension of β-galactosidase is much larger than the *E. coli* NADP-GDH subunit (M_r = 46,000).

Number of C. sorokiniana *NADP-GDH mRNA species*

To determine whether NADP-GDH mRNAs of different sizes accumulate in *C. sorokiniana* cells in which the α- and β-subunits are being simultaneously induced in 29 mM ammonium medium, total RNA was extracted from cells harvested at intervals during a 240 min induction period and analyzed by Northern blot analysis. The restriction-fragment hybridization probes were prepared from the highly conserved region (i.e., 242 bp; see Fig. 1A) and from the 3′-untranslated (non-coding) region (i.e., 378 bp, see Fig. 1A). The rationale behind the use of these two different probes, which originate from the same cDNA, is that genes which belong to multigene families are observed [11,14] to be very similar in their amino acid coding regions but tend to diverge rapidly in the sequences which encode the 5′- and 3′-untranslated regions of their mRNAs. Thus, the NADP-GDH conserved-region probe was anticipated to hybridize to all NADP-GDH mRNA species (since they would have the conserved region in common) whereas the 3′-untranslated region probe would be predicted to hybridize only to its unique NADP-GDH mRNA. The autoradiograms of the Northern blots which were analyzed with these two probes is shown in Fig. 4. Both probes hybridized to a single size (2.2 kb) NADP-GDH mRNA (Fig. 4, panels B,D). Furthermore, the same changes in abundance of NADP-GDH mRNA were observed with the two probes (Fig. 4, panels B,D) and no additional bands were detected following longer exposures of these Northern blots (Fig. 4, panel C). These results are consistent with there being only a

single species of NADP-GDH mRNA. However, the possibility that two NADP-GDH mRNAs exist of nearly identical size with coordinate kinetics of induction could not be excluded by these analyses.

Comparison of induction patterns of total NADP-GDH mRNA and catalytic activity

NADP-GDH mRNA was first detected 20 min after addition of ammonium to the cells (Fig. 4, panel C). Its concentration then increased rapidly (approximately sixteen-fold) to a maximum between 60 and 80 min (Fig. 4, panel B; Fig. 5A). After this rapid increase, the concentration of NADP-GDH mRNA decreased sharply to a minimum between 100 and 140 min and then increased once again but more slowly (Fig. 4, panel B; Fig. 5A). The same changes in NADP-GDH mRNA concentration were observed in two separate preparations of poly(A)$^+$RNA from cells harvested in this experiment, and also when total cellular RNA (containing 1 μg of poly(A)$^+$RNA per lane as determined by the ^{3}H-poly(U) binding assay) was Northern blotted (Fig. 4, panel A). Because 1 μg of poly(A)$^+$RNA was electrophoresed in each lane, the Northern blots reflect changes in relative specific-levels of NADP-GDH mRNA within a constant amount of total cellular poly(A)$^+$RNA. Therefore, these data are presented in a manner analogous to expressing concentrations of enzymes in cell extracts on a specific activity basis (e.g., mUnits enzyme/mg total cellular protein). Although a constant amount of total cellular poly(A)$^+$RNA was electrophoresed in each of the sample wells for Northern blot preparation, the actual level of total poly(A)$^+$RNA per ml of culture increased 2.8-fold in essentially a linear manner over the 240 min induction time-course (Fig. 5A). From the pattern of accumulation of total cellular poly(A)$^+$RNA per ml of culture and the relative specific-level of NADP-GDH mRNA within the total poly(A)$^+$RNA, the relative level of NADP-GDH mRNA per ml of culture was calculated and compared with the pattern of accumulation of NADP-GDH catalytic activity per ml of culture during the induction period (Fig. 5B). Even when expressed on this basis, it is evident that a net loss in NADP-GDH mRNA occurred between 80 and 100 min, indicating that both mRNA synthesis and degradation regulate the cellular level of this mRNA (Fig. 5B). There is increasing evidence that changes in rate of mRNA degradation rather than rate of gene transcription can regulate the levels of specific mRNAs in eukaryotic cells [23,34]. Therefore, it will be important to determine in future studies whether changes in rate of gene transcription or mRNA degradation are primarily responsible for the rapid fluctuation in NADP-GDH mRNA concentration during the first 120 min of the induction period in 29 mM ammonium.

The patterns of induction of unstable enzymes are predicted to parallel the patterns of accumulation of their mRNAs [36]. Although the accumulation patterns of NADP-GDH catalytic activity and mRNA per ml of culture tended to parallel each other between 0 and 40 min and also between 100 and 240 min, the sharp oscillation in NADP-GDH mRNA concentration between 40 and 100 min only resulted in small to moderate changes in the rate of accumulation of NADP-GDH catalytic activity during this same time period. This discrepancy between the two patterns suggests that some type of mRNA translational control [6,18] and/or enzyme covalent-modification/turnover [2] is preventing expression of the total hybridizable NADP-GDH mRNA as the accumulation

of a proportional amount of total NADP-GDH activity. The addition of the aforementioned findings, to the previously published model [2] for NADP-GDH regulation, indicate that a complex interplay of regulatory processes controls the ~~ammonium-induced levels/activities of the two NADP-GDH isoenzymes in the chloroplast of *C. sorokiniana*.~~

Conclusions

Several lines of evidence support the inference that the cDNA clones described herein encode a *C. sorokiniana* NADP-GDH. These cDNAs hybridize strongly to the *gdh*A gene of *S. typhimurium* and have high sequence identity to both prokaryotic and eukaryotic NADP-GDH genes (Fig. 2). When expressed in *E. coli* from the *lac* promoter, the cells accumulate an antigen recognized by antibodies against the *C. sorokiniana* NADP-GDH (Fig. 3).

No differences were detected among the 17 NADP-GDH clones that were analyzed (Fig. 1). Moreover, only one NADP-GDH mRNA size was detected on Northern blots probed with restriction fragments derived from the highly conserved region or the 3′-untranslated region of the longest cDNA (Fig. 4). These latter observations are consistent with the hypothesis that the α- and β-subunits are encoded by a single mRNA species transcribed from the single NADP-GDH gene [7]. There remains the possibility, however, that more than one mRNA species might be produced as a result of differential splicing of its large precursor-mRNA. Small differences such as the omission of one of the smaller exons [7] or differences in the region upstream of the majority of the cDNAs possibly could have gone undetected in this study.

The NADP-GDH cDNA presented in this paper represents the first GDH sequence from the plant kingdom. Preliminary experiments indicate that the gene may represent a useful probe for the isolation of NADP-GDH genes from certain higher plants (J.M. Cock and R.R. Schmidt, unpublished data). The cloning of this cDNA will facilitate characterization of the molecular mechanisms regulating expression of the NADP-GDH gene in cells cultured under different nitrogen nutritional regimes. In addition, we are particularly interested in the α-subunit, whose homohexamer has an unusually low K_m for ammonium (0.02 to 3.5 mM), for use in higher plant biotechnology.

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References

1. Austen BM, Haberland ME, Smith EL: Secondary structure predictions for the ~~NAD-specific glutamate dehydrogenase of *Neurospora crassa*. J Biol Chem 255:8001-8004 (1980).~~

2. Bascomb NF, Prunkard DE, Schmidt RR: Different rates of synthesis and degradation of two chloroplastic ammonium-inducible NADP-specific glutamate dehydrogenase isoenzymes during induction and deinduction in *Chlorella sorokiniana* cells. Plant Physiol 83:85-91 (1987).

3. Bascomb NF, Schmidt RR: Purification and partial kinetic and physical characterization of two chloroplast-localized NADP-specific glutamate dehydrogenase isoenzymes and their preferential accumulation in *Chlorella sorokiniana* cells cultured at low or high ammonium levels. Plant Physiol 83:75-84 (1987).

4. Bascomb NF, Turner KJ, Schmidt RR: Specific polysome immunoadsorption to purify an ammonium-inducible glutamate dehydrogenase mRNA from *Chlorella sorokiniana* and synthesis of full length double-stranded cDNA from the purified mRNA. Plant Physiol 81:527-532 (1986).

5. Bishop JO, Rosbash M: Polynucleotide sequences in eukaryotic DNA and RNA that form ribonuclease-resistant complexes with polyuridylic acid. J Mol Biol 85:75-86 (1974).

6. Ch'ng LLC, Shoemaker DL, Schimmel P, Holmes EW: Reversal of creatine kinase translational repression by 3′-untranslated sequences. Science 248:1003-1006 (1990).

7. Cock JM, Kim KD, Miller PW, Hutson, RG, Schmidt RR: Sequence of a nuclear gene with many introns encoding chloroplastic NADP-specific glutamate dehydrogenases in *Chlorella sorokiniana*. Plant Mol Biol, submitted (1991).

8. Cock JM, Roof LL, Bascomb NF, Gehrke CW, Kuo KC, Schmidt RR: Restriction enzyme analysis and cloning of high molecular weight genomic DNA isolated from *Chlorella sorokiniana* (Chlorophyta). J Phycol 26:361-367 (1990).

9. Czarnecka E, Edelman L, Schöffl F, Key JL: Comparative analysis of physical stress responses in soybean seedlings using cloned heat shock cDNAs. Plant Mol Biol 3:45-58 (1984).

10. Davis FC, Davis RW: Polyadenylation of RNA in immature oocytes and early cleavage of *Urechis caupo*. Develop Biol 66:86-96 (1978).

11. Dennis ES, Sachs MM, Gerlach WL, Finnegan EJ, Peacock WJ: Molecular analysis of the alcohol dehydrogenase 2 (Adh 2) gene of maize. Nucleic Acids Res 13:727-743 (1985).

12. ~~Feinberg AP, Vogelstein B: A technique for radiolabeling DNA restriction~~ fragments to high specific activity. Anal Biochem 132:6-13 (1982).

13. Feinberg AP, Vogelstein B: A technique for radiolabeling DNA restriction fragments to high specific activity (Addendum). Anal Biochem 137:266-267 (1985).

14. Gebhardt C, Oliver JE, Forde BG, Saarelainen R, Miflin BJ: Primary structure and differential expression of glutamine synthetase genes in nodules, roots and leaves of *Phaseolus vulgaris*. EMBO J 5:1429-1435 (1986).

15. Goldschmidt-Clermont M, Rahire M: Sequence, evolution and differential expression of the two genes encoding variant small subunits of ribulose bisphosphate carboxylase/oxygenase in *Chlamydomonas reinhardtii*. J Mol Biol 191:421-432 (1986).

16. Hanahan D: Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557-580 (1983).

17. Huynh TV, Young RA, Davis RW: Constructing and screening cDNA libraries in λgt10 and λgt11. In: Glover DM (ed) DNA Cloning: A Practical Approach, Vol. I. IRL Press, Oxford, England (1985).

18. Jackson RJ, Standart N: Do the poly(A) tail and 3′ untranslated region control mRNA translation? Cell 62:15-24 (1990).

19. Karlin-Neumann GA, Kohorn BD, Thornber JP, and Tobin EM: A chlorophyll a/b-protein encoded by a gene containing an intron with characteristics of a transposable element. J Mol Appl Genet 3:45-61 (1985).

20. Keegstra K, Olsen LL: Chloroplastic precursors and their transport across the envelope membranes. Annu. Rev. Plant Physiol. Plant Mol. Biol 40:471-501 (1989).

21. Kinnaird JH, Fincham JRS: The complete nucleotide sequence of the *Neurospora crassa am* (NADP-specific glutamate dehydrogenase) gene. Gene 26:253-260 (1983).

22. Maniatis T, Fritsch EF, Sambrook J: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).

23. Marzluff WF, Pandey NB: Multiple regulatory steps control histone mRNA concentrations. Trends Biochem Sci 13:49-52 (1988).

24. ~~Mattaj IW, McPherson MJ, Wootton JC: Localization of a strongly conserved section of coding sequence in glutamate dehydrogenase genes. FEBS Lett 147:21-25 (1982).~~

25. McPherson MJ, Wootton JC: Complete nucleotide sequence of the *Escherichia coli gdh*A. Nucleic Acids Res 11:5257-5266 (1983).

26. Meredith MJ, Gronostajski RM, Schmidt RR: Physical and kinetic properties of the nicotinamide adenine dinucleotide-specific glutamate dehydrogenase purified from *Chlorella sorokiniana*. Plant Physiol 61:967-974.

27. Miller ES, Brenchley JE: Cloning and characterization of *gdh*A, the structural gene for glutamate dehydrogenase of *Salmonella typhimurium*. J Bacteriol 157:171-178 (1984).

28. Mountain A, McPherson MJ, Baron AJ, Wootton JC: The *Klebsiella aerogenes* glutamate dehydrogenase (*gdh*A) gene: cloning, high-level expression and hybrid enzyme formation in *Escherichia coli*. Mol Gen Genet 199:141-145 (1985).

29. Murray EE, Lotzer J, Eberle M: Codon usage in plant genes. Nucleic Acids Res 17:477-498 (1989).

30. Nadal-Ginard B, Gallego ME, Andreadis A: Alternative splicing: mechanistic and biological implications of generating multiple proteins from a single gene. In : Setlow JE (ed) Genetic Engineering. Vol. 9. Plenum Press, New York (1987).

31. Pelham HRB, Jackson RJ: An efficient mRNA-dependent translation system from reticulocyte lysates. Eur J Biochem 67:247-256 (1976).

32. Prunkard DE, Bascomb NF, Molin WT, Schmidt RR: Effect of different carbon sources on the ammonium induction of different forms of NADP-specific glutamate dehydrogenase in *C. sorokiniana* cells cultured in light and dark. Plant Physiol 81:413-422 (1986).

33. Prunkard DE, Bascomb NF, Robinson RW, Schmidt RR: Evidence for chloroplastic localization of ammonium-inducible glutamate dehydrogenase and synthesis of its subunits from a cytosolic precursor-protein in *Chlorella sorokiniana*. Plant Physiol 81:349-355 (1986).

34. Raghow R: Regulation of messenger RNA turnover in eukaryotes. Trends Biochem Sci 12:358-360 (1987).

35. Sanger F, Miklen S, Coulson AR: DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463-5467 (1977).

~~36. Schmidt RR: Transcriptional and post-transcriptional control of enzyme levels in eucaryotic microorganisms. In: Padilla GM, Cameron IL, Zimmerman A (eds) Cell Cycle Controls, Academic Press, New York (1974).~~

37. Sharp PM, Cowe E, Higgins DG, Sheilds DC, Wolfe KH, Wright F: Codon usage patterns in *Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae, Schizosaccharomyces pombe; Drosophila melanogaster,* and *Homo sapiens*: a review of the considerable within-species diversity. Nucleic Acids Res 16:8207-8211 (1988).

38. Tabor S, Richardson CC: DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc Natl Acad Sci USA 84:4767-4771 (1987).

39. Turner KJ, Bascomb NF, Lynch JJ, Molin WT, Thurston CF, Schmidt RR: Evidence for messenger ribonucleic acid of an ammonium-inducible glutamate dehydrogenase and synthesis, covalent modification, and degradation of enzyme subunits in uninduced *Chlorella sorokiniana* cells. J Bacteriol 146:578-589 (1981).

40. Turner KJ, Gronostajski RM, Schmidt RR: Regulation of initial rate of induction of nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase during the cell cycle of synchronous *Chlorella*. J Bacteriol 134:1013-1019 (1978).

41. Valle F, Becerril B, Chen E, Seeburg P, Heyneker H, Bolivar F: Complete nucleotide sequence of the glutamate dehydrogenase gene from *Escherichia coli* K-12. Gene 27:193-199 (1984).

42. Werneke JM, Chatfield JM, Ogren WL: Alternative mRNA splicing generates the two ribulosebisphosphate carboxylase/oxygenase activase polypeptides in spinach and *Arabidopsis*. The Plant Cell 1:815-825 (1989).

43. Wootton JC: The coenzyme-binding domains of glutamate dehydrogenases. Nature 252:542-546 (1974).

44. Yanisch-Perron C, Vieira J, Messing J: Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC19 vectors. Gene 33:103-119 (1985).

45. Yeung AT, Turner KJ, Bascomb NF, Schmidt RR: Purification of an ammonium-inducible glutamate dehydrogenase and the use of its antigen affinity column-purified antibody in specific immunoprecipitation and immunoadsorption procedures. Anal Biochem 110:216-228 (1981).

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Table 1. *Codon usage for translation of a 1,572 bp open-reading frame, within a 2,145 bp consensus NADP-GDH cDNA, encoding approximately 98 percent of a* C. sorokiniana *NADP-GDH precursor protein. This table shows the number of times each amino acid codon appears in the open-reading frame.*

TTT	phe	F	2	TCT	ser	S	2	TAT	tyr	Y	2	TGT	cys	C	-
TTC	phe	F	17	TCC	ser	S	15	TAC	tyr	Y	16	TGC	cys	C	9
TTA	leu	L	-	TCA	ser	S	-	TAA	OCH	Z	1	TGA	OPA	Z	-
TTG	leu	L	-	TCG	ser	S	5	TAG	AMB	Z	-	TGG	trp	W	4
CTT	leu	L	2	CCT	pro	P	3	CAT	his	H	-	CGT	arg	R	2
CTC	leu	L	1	CCC	pro	P	17	CAC	his	H	6	CGC	arg	R	27
CTA	leu	L	1	CCA	pro	P	1	CAA	gln	Q	-	CGA	arg	R	-
CTG	leu	L	34	CCG	pro	P	6	CAG	gln	Q	26	CGG	arg	R	1
ATT	ile	I	1	ACT	thr	T	2	AAT	asn	N	1	AGT	ser	S	-
ATC	ile	I	23	ACC	thr	T	17	AAC	asn	N	19	AGC	ser	S	11
ATA	ile	I	-	ACA	thr	T	-	AAA	lys	K	-	AGA	arg	R	3
ATG	met	M	14	ACG	thr	T	2	AAG	lys	K	36	AGG	arg	R	1
GTT	val	V	4	GCT	ala	A	3	GAT	asp	D	3	GGT	gly	G	1
GTC	val	V	5	GCC	ala	A	30	GAC	asp	D	23	GGC	gly	G	45
GTA	val	V	-	GCA	ala	A	0	GAA	glu	E	-	GGA	gly	G	0
GTG	val	V	31	GCG	ala	A	15	GAG	glu	E	33	GGG	gly	G	1

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Figure Legends

Fig. 1. Restriction maps of 17 NADP-GDH cDNAs isolated from a *C. sorokiniana* cDNA library prepared from total poly(A)$^+$RNA extracted from cells induced for 80 min ~~in 29 mM ammonium medium. Panel A, 2,145 bp consensus NADP-GDH cDNA restriction map. The heavy and light black lines are the amino acid coding region and~~ the 3′-untranslated region, respectively. The regions corresponding to the conserved region probe (242 bp *Pst*I fragment) and the 3′-untranslated region probe (378 bp *Nar*I/*Sma*I fragment) are indicated. Panel B, the cDNA clones pGDc2, 3, 6, 7, 10, and 23 were isolated using a heterologous 1.2 kb probe from the *gdh*A gene from *S. thyphimurium*. Both strands of pGDc23 (1.91 kb) have been sequenced as indicated by the arrows. pGDc23 has a poly(A)tail of 70 nucleotides which is included in the length of the consensus cDNA restriction map. Panel C, the cDNA clones, pGDc30, 31, 32, 33, 34, 35, 36, 38, 39, 42 and 44 were isolated using a homologous 115 bp *Pst*I fragment from near the 5′-end (overlapping into conserved region) of pGDc23.

Fig. 2. Nucleotide sequence of the consensus *C. sorokiniana* NADP-GDH cDNA derived from the 17 cDNAs described in Fig. 1. Beginning with the first nucleotide from the 5′-terminus of this consensus cDNA, an ORF was identified that encodes a 57,401 Da polypeptide (524 amino acids) which is 98% of the molecular weight of the NADP-GDH precursor protein(s) (58,500 Da). The deduced amino acid sequence of this polypeptide (Cs) is compared with those of the *E. coli* (Ec) and *N. crassa* (Nc) NADP-GDHs. Their percent identities were calculated over the entire length of each NADP-GDH which overlapped with the *C. sorokiniana* polypeptide and also for the strongly conserved region (i.e., between the arrows) identified by Mattaj *et al.* [24].

Fig. 3. Expression of a *C. sorokiniana* NADP-GDH cDNA in *E. coli*. The pGDc23 insert was fused in-frame to a portion of the *lac* z gene in the pBluescript SK$^-$ vector which directed the synthesis of a fusion protein composed of 39 residues of the β-galactosidase followed by 440 residues of the *C. sorokiniana* NADP-GDH as shown below:

```
            39 Amino Acids            440 Amino Acids
  M          G   G   R   N   S    R   Q          V
[ATG.......GGC TGC AGG AAT TCC][CGC CAG.......GTT TAA]
                       EcoRI                     Stop
                       linker                    codon
        pBS-SK- polylinker           NADP-GDH cDNA
```

The Western blot was prepared after SDS-PAGE and probed with *C. sorokiniana* anti-NADP-GDH IgG. Lane 1, cell extract from *E. coli* strain JM109; Lane 2, cell extract from the bacterium carrying the plasmid, pBS-GDc23; Lane 3, cell extract from the bacterium carrying pBS-GDc23 after the addition of 5 mM IPTG to the culture; Lane 4, protein molecular-weight standards; Lane 5, cell extract from *C. sorokiniana* containing the NADP-GDH α-subunit.

Fig. 4. Northern blot analysis of total cellular RNA and total poly(A)$^+$RNA to estimate the number and size of mRNAs encoding the NADP-GDH α- and β-isoenzymes in *C. sorokiniana* cells induced in 29 mM ammonium medium for 240 min. A, Total cellular RNA hybridized to the 242 bp probe from the highly conserved region probe in the 1.91 kb cDNA (see Fig. 1A). B, Total poly(A)$^+$RNA hybridized to the 242 bp conserved region probe. C, Same as panel B except the autoradiogram was exposed for a longer time to show presence of mRNA by 20 min into the induction period. D, Total poly(A)$^+$RNA hybridized to the 378 bp probe from the non-coding region in the 1.91 kb cDNA (see Fig. 1A). A constant amount of poly(A)$^+$RNA from each of the harvest times was loaded onto the gels.

Fig. 5. The patterns of accumulation of NADP-GDH mRNA, total poly(A)$^+$RNA, and NADP-GDH catalytic activity in *C. sorokiniana* cells induced in 29 mM ammonium medium for 240 min. A, (●) specific level of NADP-GDH mRNA (% of poly(A$^+$)RNA); (Δ) relative level poly(A)$^+$RNA per ml. B, (●) relative level NADP-GDH mRNA per ml; (○) NADP-GDH catalytic activity per ml. The mRNA levels were obtained by scanning the 2.2 kb bands in Panel B of the Northern blot in Fig. 4 with a laser densitometer.

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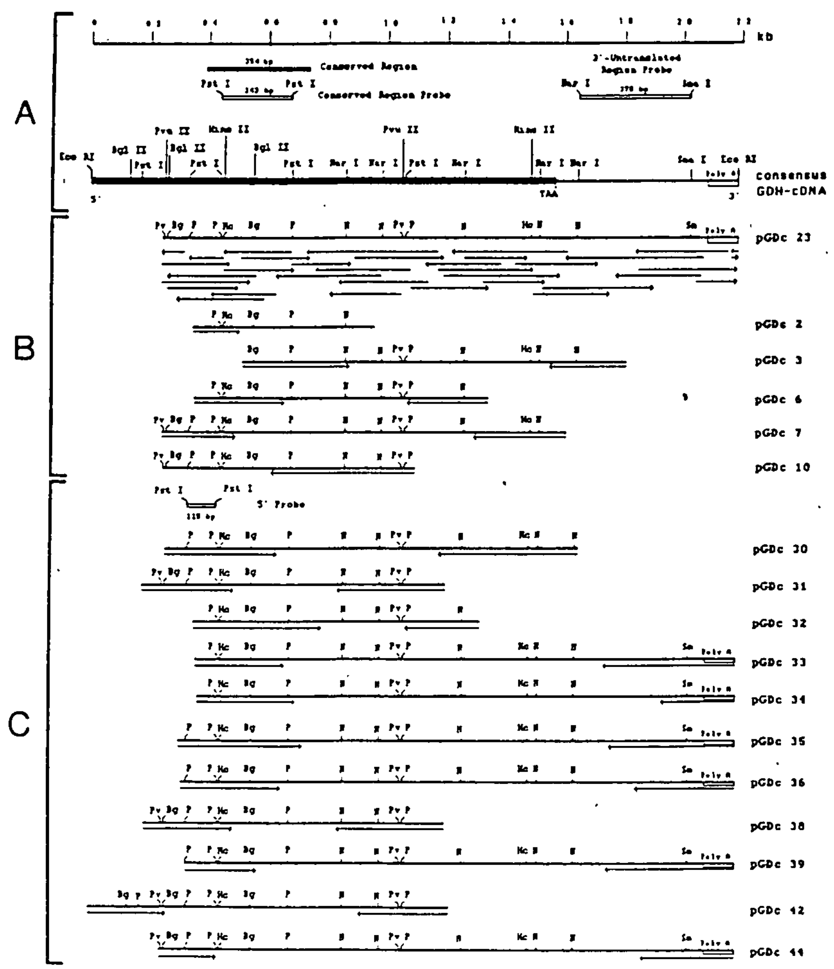
Cook et al. Fig. 1



Cock et al Fig. 2

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   TGCAGACCGCCCTCGTCGCCAAGCCTATCGTGGCCGCCCGGCCTGCGCAGCCCCGCGCTGCCTCGCGCCGTGGCCTGCGCGCGTGGTCGC 90
Cs C G P P S S P S L S W P P G L R S P A L P R A V A C A R G R

   TCCGCCAAGCGCGATGTCGCCGCCAAGCGTCTGAGGAGCAGATCTCCGCGGATGGACGCCACCACCGGCGACTTCACGGCGCTGCAGAAG 180
   S A K R D V A A K R L R S R S P R M D A T T G D F T A L Q K

   GCGGTGAAGCAGATGGCCACCAAGGCGGGCACTGAGGGCCTGGTGCACGGCATCAAGAACCCCGAGCTGCGCCAGCTGCTGACCGAGATC 270
Cs A V K Q M A T K A G T E G L V H G I K N P E L R Q L L T E I
Ec                             M D Q T Y S L . S F L N H V Q K
Nc                                                           S

   TTCATGAAGGACCCGGAGCAGCAGGAGTTCATGCAGGCGGTGCGCGAGGTGGCCGTCTCCCTGCAGCCCGTGTTCGAGAAGCGCCCCGAG 360
   F M K D P E Q Q E F M Q A V R E V A V S L Q P V F E K R P E
   R N P N Q T E F A Q A V R F V M T T L W P F L E Q N P K Y R
   N L P S E P E F E Q A Y K E L A Y T L E N S S L . Q . H . .

   CTGCTGCCCATCTTCAAGCAGATCGTTGAGCCTGAGCGCGTGATCACCTTCCGCGTGTCCTGGCTGGACGACGCCGGCAACCTGCAGGTC 450
   L L P I F K Q I V E P E R V I T F R V S W L D D A G N L Q V
   Q M S L L E R L . . . . . . Q . . . V . V . . R N Q I . .
   Y R T A L T V A S I . . . . . Q . . . V . E . . N . . V . .

   AACCGCGGCTTCCGCGTGCAGTACTCGTCCGCCATCGGCCCCTACAAGGGCGGCCTGCGCTTCCACCCCTCCGTGAACCTGTCCATCATG 540
   N R G F R V Q Y S S A I G P Y K G G L R F H P S V N L S I M
   . . A W . . . F . . . . . . . . . M . . . . . . . . . L
   . . . Y . . . F N . . L . . . . . . L . . . . . . . . L

   AAGTTCCTTGCCTTTGAGCAGATCTTCAAGAACAGCCTGACCACCCTGCCCATGGGCGGCGGCAAGGGCGGCTCCGACTTCGACCCCAAG 630
   K F L A F E Q I F K N S L T T L P M G G G K G G S D F D P K
   . . . G . . . T . . . A . . . . . . . . . . . . . . . . . .
   . . . G . . . . . . . A . . G . S . . . . . . . A . . . . .

   GGCAAGAGCGACGCGGAGGTGATGCGCTTCTGCCAGTCCTTCATGACCGAGCTGCAGCGCCACATCAGCTACGTGCAGGACGTGCCCGCC 720
   G K S D A E V M R F C Q S F M T E L Q R H I S Y V Q D V P A
   . . . E G . . . Q . . . A L . . . . Y . . L G A D T . . . .
   . . . . . . . I R . . . C A . . A . . H K . . G A D T . . . .

   GGCGACATCGGCGTGGGCGCGCGCGAGATTGGCTACCTTTTCGGCCAGTACAAGCGCATCACCAAGAACTACACCGGCGTGCTGACCGGC 810
   G D I G V G A R E I G Y L F G Q Y K R I T K N Y T G V L T G
   . . . . . . G . . V . F M A . M M . K L S N . T A C . F . .
   . . . . . . G . . . . . M . . A . R K A A N R F E . . . . .

   AAGGGCCAGGAGTATGGCGGCTCCGAGATCCGCCCCGAGGCCACCGGCTACGGCGCCGTGCTGTTTGTGGAGAACGTGCTGAAGGACAAG 900
   K G Q E Y G G S E I R P E A T G Y G A V L F V E N V L K D K
   . . L S F . . . L . . . . . . . . . L . Y . T . A M . . R H
   . . L S W . . . L . . . . . . . . . L . Y Y . G H M . E Y S

   GGCGAGAGCCTCAAGGGCAAGCGCTGCCTGGTGTCTGGCGCGGGCAACGTGGCCCAGTACTGCGCGGAGCTGCTGCTGGAGAAGGGCGCC 990
   G E S L K G K R C L V S G A G N V A Q Y C A E L L L E K G A
   . M G F E . M . V S . . . S . . . . . . A I . K A M . F . .
   . A G S YA . . . V A L . . S . . . . . . A . L K . I . L . .

   ATCGTGCTGTCGCTGTCCGACTCCCAGGGCTACGTGTACGAGCCCAACGGCTTCACGCGCGAGCAGCTGCAGGCGGTGCAGGACATGAAG 1080
   I V L S L S D S Q G Y V Y E P N G F T R E Q L Q A V Q D M K
   R . I T A . . . S . T . V D E S . . . K . K . A R L I E I .
   T . V . . . . . K . . A L V A T G E S G I T V E D I N A V . A

   AAGAAGAACAACAGCGCCCGCATCTCCGAGTACAAGAGCGACACCGCCGTGTATGTGGGCGACCGCCGCAAGCCTTGGGAGCTGGACTGC 1170
   K K N N S A R I S E Y K S D T A V Y V G D R R K P W E L D C
   A S R D RD G . V A D . A K E FG L V Y - L E G Q Q . . S . P -
   I . E - - . . Q . L T S F Q H . GH LK W I E G A R . . L H V G

   CAGGTGGACATCGCCTTCCCCTGCGCCACCCAGAACGAGATCGATGAGCACGACGCCGAGCTGCTGATCAAGCACGGCTGCCAGTACGTG 1260
   Q V D I A F P C A T Q N E I D E H D A E L L I K H G C Q Y V
   - . . . . . . L . . . . . . L . V D A . H Q . . A N . V K A .
   K . . . . L . . . . . . . . V S K E E . . G . L A A . . K F .

   GTGGAGGGCGCCAACATGCCCTCCACCAACGAGGCCATCCACAAGTACAACAAGGCCGGCATCATCTACTGCCCCGGCAAGGCGGCCAAC 1350
   V E G A N M P S T N E A I H K Y N K A G I I Y C P G K A A N
   A . . . . . . T . I . . . T E L F Q Q . . V L F A . . . . . .
   A . . S . . G C . L . . . . E VFENNRKE . K . EAVW . A . . . . . .

   GCCGGCGGCGTGGCGGTCAGCGGCCTGGAGATGACCCAGAACCGCATGAGCCTGAACTGGACTCGCGAGGAGGTTCGCGACAAGCTGGAG 1440
   A G G V A V S G L E M T Q N R M S L N W T R E E V R D K L E
   . . . . . T . . . . . A . . A A R . G . K A . K . D A R . H
   C . . . . . . . . . . A . . S Q R . . . . . Q A . . D E . . K

   CGCATCATGAAGGACATCTACGACTCCGCCATGGGGCCGTCCCGCGAGTACAATGTTGACCTGGCTGCGGGCGCCAACATCGCGGGCTTC 1530
   R I M K D I Y D S A M G P S R E Y N V D L A A G A N I A G F
   H . . L . . H H A C V E H G G . GE Q T N Y V Q . . . . . . . .
   D . . . N A F F N G L N T A K T Y V E A A E G E L P S . V . . S . . . . . .

   ACCAAGGTGGCTGATGCCGTCAAGGCCCAGGGCGCTGTTTAAGCTGCCCAGGCCCAAGCCACGGCTCACCGGCAATCCAACCCAACCAAC 1620
   T K V A D A V K A Q G A V *
   V . . . . . M L . . . V I *
   V . . . Q . M H D . . D W W S K N *

   TCAACGGCCAGGACCTTTTCGGAAGCGGCGCCTTTTTCCCAGCCAGGGCCCTCACCTGCCCTTTCATAACCCTGCTATTGCCGCCGTGCC 1710
   CCTGCAATTCCACCCCAAGAAGAACTAGCGGCACTTGACTGCATCAGGACGGCTATTTTTTTCGCGACGCGCGCTCACCCCGAGAGCCTC 1800
   TCTCCCCCGAGCCCTAAGCGCTGACGTCCGCCCGACTTTGCCTCGCACATCGCTCGGTTTTGACCCCCTCCAGTCTACCCACCCTGTTGT 1890
   GAAGCCTACCAGCTCAATTGCCTTTTAGTGTATGTGCGCCCCCTCCTGCCCCCGAATTTTCCTGCCATGAGACGTGCGGTTCCTAGCCTG 1980
   GTGACCCCAAGTAGCAGTTAGTGTGCGTGCCTTGCCCTGCGCTGCCCGGGATGCGATACTGTGACCTGAGAGTGCTTGTGTAAACACGAC 2070
   GAGTC (Poly-A)70                                                                           2145
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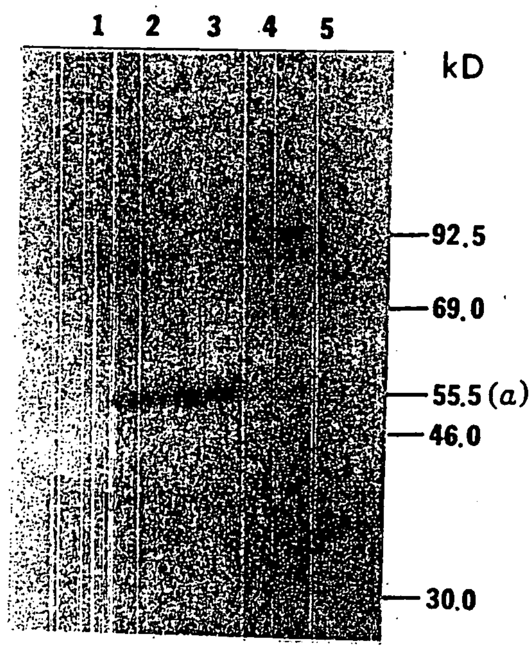
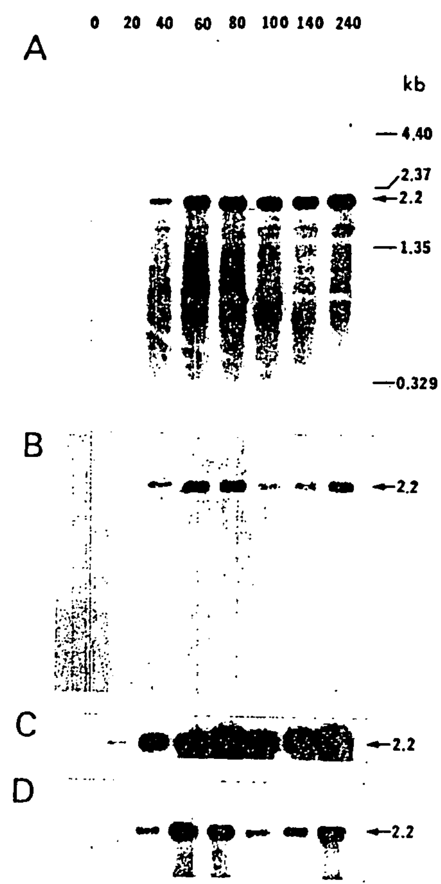
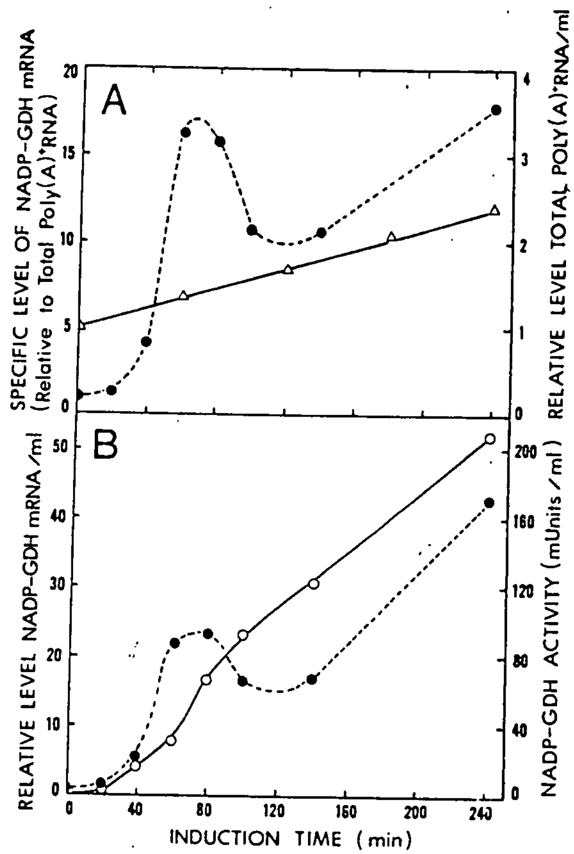
Cook et al. Fig. 3



Cook et al. Fig. 4



Cock et al. Fig. 5



#2

A Nuclear Gene with Many Introns Encoding Chloroplastic NADP-Specific Glutamate Dehydrogenases in *Chlorella sorokiniana*

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Running Title: Glutamate dehydrogenase gene sequence

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Accession number: ______

Key words: *Chlorella sorokiniana*, exons, gene sequence, glutamate dehydrogenase, introns, NADP-specific

Abstract

A probe derived from the highly conserved region of a *Chlorella sorokiniana* NADP-specific glutamate dehydrogenase (NADP-GDH) cDNA was used to select eight NADP-GDH clones from a *C. sorokiniana* genomic DNA library. Restriction maps of the four longest overlapping genomic clones showed them to span a 21.9 kb region. Almost 10 kb of this region were sequenced and shown to contain the complete 2145 bp consensus sequence which encodes 98% of the 58,500 Da precursor-protein(s) from which the NADP-GDH α- and β-subunits are processed. The consensus sequence is distributed among 22 exons, ranging in size from 18 bp to 550 bp, covering 7143 bp. The 21 introns in this gene are an unusually large number for an eukaryotic microorganism. To determine whether this sequence corresponds to the only NADP-GDH gene in this organism, Southern blot analyses were performed on restriction fragments produced by digestion of the genomic clones and total cellular genomic DNA with several endonucleases. The restriction fragments were hybridized to probes corresponding to sequences from the highly conserved and 3′-untranslated regions in the NADP-GDH consensus cDNA. The rationale was that the former probe should hybridize to any NADP-GDH gene whereas the latter probe to only the unique NADP-GDH gene having the untranslated region. Although all of the Southern blot data are consistent with *C. sorokiniana* having only one NADP-GDH gene, very convincing evidence came from *Sma*I digests which produced only a single 6.9 kb fragment which hybridized strongly with both probes.

Introduction

Chlorella sorokiniana cells have been shown to have ammonium-inducible NADP-specific glutamate dehydrogenase (NADP-GDH) isoenzymes that are chloroplast-localized [3,4,29,30]. Depending upon the nitrogen-nutritional conditions, the total chloroplastic NADP-GDH activity can be associated with homohexamers composed of either α- or β-subunits (55,500 Da or 53,000 Da, respectively) or with a mixture of homo- and heterohexamers (i.e., 6α, 5α:1β, 4α:2β, 3α:3β, 2α:4β; 1α:5β, 6β) [4,29].

Although the α- and β-homohexamers have strikingly different affinities for ammonium [4], and their *in vivo* rates of turnover are very different [3], they have very similar peptide maps [4] and are derived from precursor proteins [4,5,30] of identical size (58,500 Da). Antibodies prepared against the β-homohexamer were shown to immunoprecipitate both isoenzymes [3,4,42]. Because these latter properties indicate that the α- and β-subunits have a high degree of sequence homology, Cock *et al.* [9] attempted to determine whether these subunits are encoded by one or two NADP-GDH mRNAs, i.e., encoding a single precursor-protein which is differentially processed to yield α- or β-subunits, or two precursor-proteins of essentially the same size which are processed specifically to yield the two types of subunits, respectively. They prepared a cDNA library from *C. sorokiniana* cells which were simultaneously synthesizing NADP-GDH α- and β-subunits. Seventeen NADP-GDH cDNAs with identical overlapping restriction-maps/nucleotide sequences (0.6 to 1.91 kb) were isolated. No NADP-GDH cDNAs with different sequences were isolated. Each cDNA contained a 354 bp conserved region [23], with a deduced amino acid sequence, having 77% and 73% identity with the corresponding regions in the *Escherichia coli* [24,38] and *Neurospora crassa* [22] NADP-GDHs. From the 17 cDNAs, a 2145 bp consensus sequence was derived which contains an open-reading-frame (ORF) for a 57,401 Da protein which is 98% of the size of the precursor-protein(s) from which the two subunits are processed. The 57,401 Da protein appears to contain part of the chloroplast transit-peptide and sufficient sequence for the α- or β-subunit, if processing of a single precursor-protein can give rise to either type of subunit. To determine whether the two subunits are encoded by NADP-GDH mRNAs of different size or with different patterns of induced accumulation, these workers performed Northern blot analyses on total cellular RNA and poly(A)$^+$RNA isolated from cells during a 240 min induction period in which both subunits were accumulating. The blots were hybridized separately with cDNA probes derived from the conserved region and the 3′-untranslated region of the longest (1.91 kb) NADP-GDH cDNA. The rationale behind the use of the two probes was that the amino acid coding regions of closely related genes are observed [11,14] to remain relatively stable whereas the 3′- and 5′-untranslated regions tend to diverge rapidly. Therefore, the former probe was anticipated to hybridize to any NADP-GDH mRNA having the highly conserved region and the latter probe was predicted to hybridize only to the unique mRNA from which it was derived. Using either probe, the same pattern of accumulation of a single-size (2.2 kb) mRNA was seen throughout the induction period. These observations were taken as evidence that the α- and β-subunits are encoded by a single mRNA.

The purpose of the research described in this paper was to isolate and sequence the gene corresponding to the NADP-GDH consensus cDNA sequence described by Cock *et al.* [9], and to perform Southern blot analyses with the highly conserved and 3′-

untranslated region probes to determine whether the *C. sorokiniana* genome contains multiple NADP-GDH genes. The data presented herein are consistent with this organism possessing only a single NADP-GDH gene having high homology with the conserved region probe.

Materials and methods

Preparation and screening of a C. sorokiniana *genomic DNA library*

~~*C. sorokiniana* cells were cultured asynchronously in continuous light in nitrate medium~~ which was aerated with 2% CO_2-air at 38.5°C as previously described [29,37]. The cell suspension was harvested, 250 ml aliquots were decanted into 500 ml Erlenmyer flasks, and stored without aeration at a low light intensity (i.e., 40.4 μEm^2s^{-1}) at 25°C until extraction of genomic DNA. High molecular weight genomic DNA (approximately 70 kb) was extracted from the unfrozen cells by the procedure of Cock *et al.* [10]. These workers showed that freezing of the cells before extraction of the DNA results in the recovery of lower molecular weight DNA (20 to 30 kb).

The purified *C. sorokiniana* DNA was sent to Clontech Laboratories (Palo Alto, CA) for custom synthesis of primary and amplified genomic libraries in λEMBL3 as discussed earlier [10]. These libraries contained 2.5 x 10^5 and 1.5 x 10^{10} pfu, respectively.

From the amplified library, 1.8 x 10^6 pfu were probed in three rounds of screening with a 242 bp *Pst*I fragment from the highly conserved coding-region of the *C. sorokiniana* NADP-GDH cDNA clone, pGDc23 [9]. After digestion of pGDc23 with *Pst*I, the 242 bp fragment was separated from other fragments by agarose-gel (low melting point agarose, Bethesda Research Laboratories) electrophoresis, and then recovered from the gel by electroelution. A radiolabelled probe (1.8 x 10^9 cpm/1.5 μg) was synthesized from this fragment by use of random hexamer primers (Pharmacia) as previously described [9,12].

After the third screening, the phage, carrying putative NADP-GDH genomic-DNA inserts, were replicated in top-agar plate cultures and recovered by immunoadsorption using LambdaSorb (Promega). The DNA was extracted from these phage and their insert DNAs were released by *Sal*I digestion. These *Sal*I fragments were subcloned into *Sal*I digested pUC18 and transformed into *E. coli* JM109 [15,41]. These subclones were designated the pGDg series.

Sequencing of NADP-GDH genomic DNA clones

Four of the longest NADP-GDH genomic clones, pGDg 8.4.4, pGDg 14.10.1, pGDg 14.4.1, and pGDg 15.2.1, were analyzed by restriction mapping. Restriction fragments and exonuclease III/mung bean nuclease deletion fragments from the overlapping clones pGDg 8.4.4 and pGDg 14.10.1 were prepared and subcloned into M13 vectors [41] mp 18/19 for sequencing by the dideoxy method [32] using modified T7 DNA polymerase (Sequenase, United States Biochemical Corp.).

Southern blots

Restriction fragments were electrophoresed in 0.8% agarose gels, blotted [34] onto nylon membranes [31] (Hybond N, Amersham), and prehybridized for approximately 5 h in 10 ml of 20% formamide, 0.6 M NaCl, 0.06 M sodium citrate, 0.01 M EDTA, 0.1% SDS, 5 x Denhardt's solution, 50 μg/ml sonicated/boiled calf thymus DNA. Hybridization was performed at room temperature for approximately 16 h in 5 ml of 50% formamide, 10%

dextran sulfate, 1 x Denhardt's solution, 4 x SSC, 0.1 M EDTA, 0.1% SDS, and 25 μg/ml of sonicated/boiled calf thymus DNA to which the denatured radiolabelled cDNA probe was added. When restriction fragments were used as hybridization probes, they were all derived from the *C. sorokiniana* NADP-GDH cDNA clone, pGDc23 [9]. The entire ~~pGDc7 cDNA [9] was also used as a hybridization probe. Probes were radiolabelled as described above for screening the genomic library. The membranes were washed three~~ times for 20 min at high stringency conditions (0.1 x SSC, 0.1% SDS, 65°C). Autoradiography was performed at -70°C with Kodak X-Omat AR film.

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Results and discussion

Restriction maps of C. sorokiniana *NADP-GDH genomic clones*

~~The *C. sorokiniana* genomic library in λEMBL3 was screened with a 242 bp hybridization probe derived from the 354 bp highly conserved region [23] of the *C. sorokiniana* 1.91 kb~~ NADP-GDH cDNA, pGDc23 [9]. As reported earlier [9], the conserved region of pGDc23 has high identity with the corresponding regions in both bacterial and fungal NADP-GDH genes. From the genomic-library screening, eight putative NADP-GDH clones were isolated. Comparison of the restriction maps of these clones showed them to be derived from the same region of the *C. sorokiniana* genome. Restriction maps of the four longest overlapping genomic DNA inserts (i.e., pGDg 14.10.1, 8.4.4, 14.4.1, and 15.2.1) are aligned in Fig. 1. Together they encompass a 21.9 kb region of the genome.

C. sorokiniana total genomic DNA and the aforementioned four putative NADP-GDH genomic clones in EMBL3 were digested separately with *Pvu*II. The resulting restriction fragments derived from the five DNA preparations were subjected to Southern blot analysis using pGDc7 as the hybridization probe (Fig. 2A,B). This 1347 bp NADP-GDH cDNA is identical to pGDc23 except that it is missing the poly(A)tail and most of the 3′-untranslated region. Under low and high stringency conditions, this cDNA probe was observed to hybridize to the same size restriction fragments in digests of the total *C. sorokiniana* genomic DNA and of the longest NADP-GDH genomic clones, pGDg 14.10.1 and 14.4.1 (Fig. 2A,B). However, the digests of the two shorter clones, pGDg 8.4.4 and 15.2.1, each yielded only a single hybridizing fragment, indicating that these clones are missing a portion of the NADP-GDH gene.

Sequence of a C. sorokiniana *NADP-GDH gene and identification of exons/introns*

A 9872 bp region of genomic DNA was sequenced in both directions from overlapping regions of pGDc 14.10.1 and 8.4.4 (Fig. 1). When this sequence was compared with the *C. sorokiniana* 2145 bp consensus NADP-GDH cDNA sequence, derived from the sequences of 17 NADP-GDH cDNA clones [9], the position of an approximately 7-8 kb NADP-GDH gene containing at least 22 exons was revealed (Fig. 3). The exons in this gene exhibit a range of lengths from 18 bp to a large exon of 550 bp at the 3′ end. The 18 bp exon is smaller than the smallest exons identified in higher plant genes [35]. However, smaller exons (i.e., 17 bp) have been observed in vertebrate [2] genes. Although the entire NADP-GDH consensus cDNA sequence can be accounted for within the 22 exons of this NADP-GDH gene, the consensus sequence represents only 97.5% of the full-length NADP-GDH mRNA [9] (i.e., 2145 bp versus 2220 bp, respectively). The remaining approximately 55 bp of unidentified sequence, corresponding to the 5′-terminus of the full-length cDNA, may prove to be part of the first exon (Fig. 3) or it possibly may be associated with new upstream exons. Therefore, we are uncertain about the actual size of the first exon which is tentatively indicated as being 17 bp in Fig. 3. As shown, the first 5 bp of this exon has the sequence TGCAG. Although this sequence is at the 5′-terminus of the consensus cDNA, it is also the sequence found at the 3′-splice sites of introns in this gene. Thus, the actual size of the exon could be 12 bp.

Alternatively, because there is another TGCAG sequence located 298 bp upstream of the one in question, the latter one could be the 5′-splice site of the twenty-third exon and the former one could be a continuation of the sequence of a larger exon.

The examination of the sequence upstream of the first exon has not resulted in the ~~identification of putative eukaryotic promoter elements. These elements may prove to be positioned upstream of the region that has been sequenced. For example, the quail~~ skeletal muscle troponin I gene has its TATA box located approximately 2 kb upstream from the exon encoding the ATG start-codon [2]. Of the approximately 10 kb that we have sequenced, only 1315 bp remains upstream from the first exon (Fig. 3) and the exon which contains the ATG start-codon has not been located yet.

Because most genes of lower eukaryotes contain very few introns, the 21 introns that have been identified so far in this NADP-GDH gene represent an unusually high number for a lower eukaryotic microorganism. For example, the majority of *Saccharomyces* genes [13,21,43] and 18% of fungal genes in general [17] are intronless. Those lower eukaryotic genes which do contain introns usually contain only one or two on the average [18].

The introns identified in the NADP-GDH gene are between 131 and 402 bp, with a mean length of 241 bp. This mean length is similar to that calculated (i.e., 249 bp) in a survey of higher plant introns [17]. The introns of fungal, insect, and vertebrate genes tend to be shorter than those of plant genes [17]. In *C. sorokiniana* gene, we have derived a consensus $\overset{C}{\underset{A}{\mathrm{AG}}}|\mathrm{GTG}\ \overset{A}{\underset{C}{\mathrm{G}}}\overset{TC}{\underset{CG}{\mathrm{G}}}$ sequence of the 5′-intron splice using the criteria suggested by Cavener [8]. This consensus closely matches the general consensus, CAG | GTAAGT [18], seen in animals and higher plants, apart from the predominance of G at position +3. Three of the 21 5′-splice sites which have been identified in this NADP-GDH gene contain C at position +2 and hence do not conform with the GT-AG rule of Breathnach and Chambon [6]. Only 19 out of 3294 introns examined in a survey by Jacob and Gallinaro [18] were found not to contain a T at position +2 of the 5′ splice site, and the only plant gene which has been identified to contain a substitution of this kind is the nodulin-24 gene of soybean [20].

Twenty NADP-GDH gene introns have a pyrimidine-rich stretch, as defined by Hanley and Schuler [16], between -3 and -20 upstream of the 3′ splice site. Introns in animal genes commonly contain a series of pyrimidines in this region [26]. Although many introns in plant genes contain this pyrimidine rich region, particularly introns of monocotyledonous plants, the introns in plant genes are in general more variable than those of animals with respect to this pyrimidine-rich region [16]. We have derived a consensus sequence of $\mathrm{C}\frac{\mathrm{TTTTTTT}}{\mathrm{CCCCCCC}}\mathrm{CcCtt}\frac{\mathrm{T}}{\mathrm{C}}\frac{\mathrm{T}}{\mathrm{C}}\mathrm{GCAG/}$ for the 3′ intron splice site of the *C. sorokiniana* NADP-GDH gene. The strong preference (i.e., 19 out of 21 introns) for G at position -4 is typical of plant introns [7] whereas mammalian introns show no preference for any nucleotide at this position.

The putative TAA stop-codon for the *C. sorokiniana* NADP-GDH consensus cDNA-sequence [9] resides in the last exon along with the entire 3′-untranslated region (Fig. 3). The region immediately downstream of this stop codon was compared with the

consensus sequence derived for this region in plant genes [19]. The *C. sorokiniana* sequence contains the conserved G and T at +1 and +3, respectively, but lacks the AT-rich region immediately downstream. The AATAAA sequence which acts as a ~~polyadenylation signal in the majority of animal and viral genes [28] is not present in the vicinity of the genomic sequence corresponding to the polyadenylation site (Fig. 3) in the~~ *C. sorokiniana* NADP-GDH consensus cDNA. Although the NADP-GDH gene lacks the AATAAA signal sequence, Sauer and Tanner [33] have shown that the cDNA encoding the H^{+}/hexose cotransporter of *C. kessleri* contains this sequence beginning 342 bp from the polyadenylation site. The closest match to this sequence in the *C. sorokiniana* NADP-GDH gene is TGTAAA which begins 17 bp upstream of the polyadenylation site; however, this latter sequence lacks the conserved nucleotides immediately downstream which were identified in a survey of higher plant polyadenylation signals [19]. Moreover, because the majority of higher plant genes lack the AATAAA motif [19], the absence of this particular polyadenylation signal in the *C. sorokiniana* NADP-GDH gene should not be considered as unusual.

Evidence for a single species of NADP-GDH gene in C. sorokiniana

To determine how many different NADP-GDH genes might be encoded in the *C. sorokiniana* genome, the genomic DNA was digested with several endonucleases and the resulting restriction fragments were subjected to Southern blot analysis using cDNA probes which hybridize to the highly conserved and 3′-untranslated regions of pGDc23 [9]. Cock *et al.* [10] showed that *C. sorokiniana* genomic DNA is highly methylated (i.e., 5.1 mol % 5-methylcytosine) and is resistant to digestion by many endonucleases. Of the 20 endonucleases that these workers tested, *Ava*II, *Pst*I, *Pvu*II, and *Taq*I digested high molecular weight *C. sorokiniana* more completely than the others. Therefore, with the realization that even certain of these four endonucleases (e.g. *Ava*II) might be inhibited by 5-methylcytosine in their restriction sites, all four were selected for use in the Southern blot analyses. The 242 bp highly conserved region probe was predicted to hybridize to restriction fragments containing this region from any species of the NADP-GDH gene in the *C. sorokiniana* genome. In contrast, because even closely related genes, which have high homologies among their amino acid coding regions, appear to diverge rapidly in their untranslated regions [11,14], the 378 bp 3′-untranslated region probe was anticipated to detect restriction fragments derived from only the unique NADP-GDH gene encoding this untranslated region.

When the Southern blots were hybridized separately with each probe and then washed under stringent conditions, the 3′-untranslated region probe detected only a single hybridizing band in the genomic DNA digests with each of the four endonucleases (Fig. 4A). The conserved-region probe also hybridized strongly to a single restriction fragment in each of the digests; however, in some of the digests, one or two weakly hybridizing bands were also visible (Fig. 4B). From the region of genomic DNA that has been sequenced, it was possible to generate restriction maps for each of the four endonucleases and to predict the number and size of restriction fragments that should hybridize to the two probes (Fig. 5). A comparison of the number and size of the predicted hybridizing-fragments (Fig. 5), with those actually observed in the Southern

blot analyses (Fig. 4A,B), showed that in some cases more fragments were observed than predicted. The extra restriction-fragments, which hybridized with the conserved region probe, appear to result in part from the entire 354 bp conserved region in the NADP-GDH cDNA being split into six exons within the gene (encompassing a 2.09 kb length of genomic DNA; Fig. 3) with restriction sites for certain of the four endonucleases residing between some of these exons. The 242 bp conserved-region probe is predicted to hybridize to all or parts of five of the six exons, spanning a 1390 bp region of genomic DNA (Fig. 5). In contrast, the entire 3′-untranslated region resides within a single exon; therefore, the 3′-untranslated region probe is predicted to hybridize to only a 378 bp region in the genomic DNA (Fig. 5).

With the exception of *Pvu*II, the other endonucleases were predicted to yield multiple restriction fragments which should hybridize with the conserved region probe. However, the *Pvu*II digest produced two hybridizing fragments: a strongly hybridizing fragment with the predicted size of 2736 bp and a weakly hybridizing fragment of approximately 3.5 kb. This latter hybridizing fragments (3477 bp) is one of several fragments that would be predicted to transiently accumulate as the *Pvu*II digestion of this region of the genomic DNA proceeds towards completion (Fig. 5). The *Pst*I digestion yielded a strongly hybridizing fragment of approximately 1100 bp with the conserved region probe. This fragment size was predicted along with smaller fragments of 342 bp and 576 bp. Overexposure of the autoradiogram (not shown) revealed the presence of these smaller fragments in both the *Pst*I and *Pvu*II:*Pst*I digest. The weakly hybridizing 2 kb fragment appears to be one of the predicted intermediates during *Pst*I digestion. The absence of this 2 kb fragment by the end of the digestion with both *Pvu*II and *Pst*I further supports the conclusion that this fragment is a partial digestion product. The *Taq*I digestion yielded the predicted 1159 bp and 590 bp hybridizing fragments with the conserved-region probe. Whereas digestion with *Ava*II was predicted to produce 1330 bp and 554 bp hybridizing fragments, the actual hybridization pattern showed a strong band at approximately 2.2 kb and weak bands at approximately 1.3 kb and 1.5 kb. Although 2211 bp and 1456 bp hybridizing fragments are predicted to be transient products, and the 1330 bp fragment an end-product, the largest fragment was actually the predominant product. The slowness by which certain *Ava*II restriction sites were cleaved may be due to the presence of 5-methylcytosine in these sites.

The number and sizes of the *Pvu*II, *Pst*I, and *Taq*I restriction fragments, which were observed (Fig. 4A) to hybridize with the 3′-untranslated region probe, were essentially identical to those which were predicted in Fig. 5. However, the sizes of the predicted and observed *Ava*II restriction fragments were not in agreement (i.e., 1059 bp versus ~ 1.7 kb). If 5-methylcytosine within the pair of closely situated *Ava*II sites located at the 3′-terminus of the 1059 bp predicted fragment (Fig. 5) prevented cutting at these sites, the next *Ava*II cleavage-site downstream would generate a 1776 bp fragment. This latter fragment is essentially the size of the one detected on the Southern blot (Fig. 4A). It is interesting to note that the closely situated pair of *Ava*II sites at the 5′-terminus of the predicted 1330 bp conserved-region hybridizing fragment also appears to be partially resistant to cleavage. Thus, although the observed patterns of hybridization of the restriction fragments with the two probes varied in some cases from the patterns predicted for complete digestion at restriction cleavage sites (identified by gene sequence

analysis), we have been able to provide a reasonable accounting for the discrepancies from the predicted patterns. Taken collectively, the aforementioned results are consistent with this organism containing a single species of NADP-GDH gene. ~~Moreover, the observation (Fig. 2) that *PvuII* digests of the total *C. sorokiniana* genomic DNA and of the longest NADP-GDH genomic clones (i.e., pGDg 14.10.1 and pGDg 8.4.4)~~ gave essentially the same pattern of hybridization with the 1.3 kb NADP-GDH cDNA, pGDc 7, can be taken as additional evidence that this organism contains a single expressed NADP-GDH gene.

As discussed above, interpretation of the Southern blot data (Fig. 4A,B), obtained with the two probes, was complicated by the presence of multiple restriction sites between the exons and also by uncomplete digestion at some of these sites. To circumvent this type of problem, the genomic DNA was digested with *Sma*I which does not cleave within the regions where the two probes hybridize. Because there are no *Sma*I sites between the conserved region and the 3′-untranslated region, cleavage of the genomic DNA with *Sma*I was predicted to produce a 6858 bp restriction fragment which should hybridize to both probes. Moreover, if there is only a single species of NADP-GDH gene in this organism, the 6958 bp fragment should be the only *Sma*I restriction fragment which hybridizes with the conserved region probe. The *Sma*I sites are positioned on the restriction maps of the four clones in Fig. 1. Those *Sma*I sites which are predicted to produce the 6958 bp fragment correspond to positions 1549 bp and 8407 bp in the sequenced region of the genomic DNA shown in Fig. 5.

In Fig. 6, a 6.9 kb restriction fragment which hybridizes strongly with both probes was indeed observed in the *Sma*I digest of genomic DNA. A *Taq*I genomic digest was performed as a control and the conserved-region and 3′-untranslated region probes detected the predicted number and size of hybridizing fragments (i.e., 1159 bp and 590 bp; and 1332 bp, respectively). A very large (> 23.1 kb) *Sma*I restriction fragment was detected which hybridized very weakly with the conserved-region probe and not at all with the other probe (Fig. 6). The weakly hybridizing fragment actually electrophoresed only a short distance beyond the position of undigested genomic DNA in the gel (Fig. 6). It should be noted that, in a separate gel (not shown), the undigested genomic DNA was shown to hybridize strongly with the conserved region probe before *Sma*I digestion. However, no hybridization was detected at its original position in the gel after digestion with *Sma*I.

Inasmuch as the conserved regions of NADP-GDH genes from bacteria [24,38], fungi [22], and *Chlorella* have high sequence homology and the α- and β-subunits of the *C. sorokiniana* were shown [4] by peptide mapping to have very high sequence homology, it seems doubtful that the large weakly hybridizing *Sma*I fragment is derived from a NADP-GDH gene encoding the α- or β-subunits. Since the NADP-GDH conserved region has been proposed [1,40] to encode a protein-domain involved in the binding of dicarboxylic acids (e.g., glutamate), certain enzymes (e.g., mitochondrial NAD-GDH [25], glutamine synthetase [14,35], transaminases, etc.) which also use dicarboxylic acid substrates may have enough sequence homology to bind the NADP-GDH conserved region probe very weakly.

Conclusions

We have accounted for the entire 2,145 bp consensus nucleotide sequence, derived from 17 NADP-GDH cDNAs by Cock *et al.* [9], within 22 exons spanning 7143 bp of the *C. sorokiniana* genome. The patterns of hybridization of the conserved-region probe and the 3'-untranslated region probe, obtained by digestion of the *C. sorokiniana* total genomic DNA with several restriction endonucleases, are consistent with this organism having only a single type of NADP-GDH gene. If there is another *C. sorokiniana* NADP-GDH gene that was not detected in the isolation of genomic clones nor in the Southern blot analyses, the sequence of its conserved region would have to be very different from any described so far for bacteria [24,38] and fungi [22] or the *C. sorokiniana* NADP-GDH gene described in this paper.

The finding of a single type of NADP-GDH gene in this organism might seem to provide additional proof for their being only a single mRNA encoding the NADP-GDH α- an β-subunits as proposed by Cock *et al.* [9]. However, the large size of this gene with its many exons provides the possibility that mRNA heterogeneity might be produced by alternative splicing [27,39] of a precursor mRNA. An important consideration is that some of these exons are very small (i.e., 18 to 54 bp) and correspond to regions in the consensus NADP-GDH cDNA which were not overlapped by all of the cDNAs isolated by Cock *et al.* [9]. For example, of 17 which they isolated and characterized, six of them (i.e., pGDc 2,6,10,31,32, and 38) were truncated and terminated between the conserved region and the beginning of the 3'-terminus of the 3'-untranslated region which is encoded in a single and the last exon in the NADP-GDH gene described herein. If the small 18 bp exon, which resides between these two regions, were to be eliminated by alternative splicing of the precursor mRNA, it is doubtful that two mRNAs differing in size by only 18 bp could be resolved by the standard electrophoresis/Northern blot procedure used in the previous study. Moreover, there are also some small exons that encode amino acid sequences that reside immediately upstream from the conserved region, and not all of the isolated cDNAs were long enough at their 5'-termini to include these exon sequences. Therefore, although the data in the present paper establish reasonably firmly that *C. sorokiniana* has a single species of NADP-GDH gene, the complexity of its structure has shown us the importance of examining the sequences of a large number of full-length cDNAs before it can be stated with certainty whether the α- and β-subunits are encoded by one or two mRNAs. To answer this question, cDNA libraries are currently being prepared from poly(A)$^+$RNA which was extracted from cells growing under conditions [4] for induction of only the α- or β-subunit. Even if two mRNAs are formed by alternative splicing, only a very limited number of exons can be deleted and still yield two RNAs that will co-electrophoreses as a 2.2 kb band. Moreover, the observation by Cock *et al.* [9] that, during the simultaneous induction of both types of subunits, the same induction pattern of the 2.2 kb mRNA band was obtained, with either the highly conserved region probe or the 3'-untranslated region probe, is consistent with the transcription of a single gene to yield one precursor-mRNA. However, as discussed above, additional research is required to establish with certainty how this precursor mRNA is processed.

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References

1. Austen BM, Haberland ME, Smith EL: Secondary structure predictions for the NAD-specific glutamate dehydrogenase of *Neurospora crassa*. J Biol Chem 255:8001-8004 (1980).

2. Baldwin AS, Kittler ELW, Emerson CP: Structure, evolution, and regulation of a fast skeletal muscle troponin I gene. Proc Natl Acad Sci USA 82:8080-8084 (1985).

3. Bascomb NF, Prunkard DE, Schmidt RR: Different rates of synthesis and degradation of two chloroplastic ammonium-inducible NADP-specific glutamate dehydrogenase isoenzymes during induction and deinduction in *Chlorella sorokiniana* cells. Plant Physiol 83:85-91 (1987).

4. Bascomb NF, Schmidt RR: Purification and partial kinetic and physical characterization of two chloroplast-localized NADP-specific glutamate dehydrogenase isoenzymes and their preferential accumulation in *Chlorella sorokiniana* cells cultured at low or high ammonium levels. Plant Physiol 83:75-84 (1987).

5. Bascomb NF, Turner KJ, Schmidt RR: Specific polysome immunoadsorption to purify an ammonium-inducible glutamate dehydrogenase mRNA from *Chlorella sorokiniana* and synthesis of full length double-stranded cDNA from the purified mRNA. Plant Physiol 81:527-532 (1986).

6. Breathnach R, Chambon P: Organization and expression of eukaryotic split genes coding for proteins. Ann Rev Biochem 50:349-383 (1981).

7. Brown JWS: A catalogue of splice junction and putative branch point sequences from plant introns. Nucleic Acids Res 14:9549-9559 (1986).

8. Cavener DR: Comparison of the consensus sequence flanking translational start-sites in Drosophila and vertebrates. Nucleic Acids Res 15:1353-1361 (1987).

9. Cock JM, Kim KD, Miller PW, Schmidt RR: Sequence and ammonium induction pattern of a mRNA encoding chloroplastic NADP-specific glutamate dehydrogenase(s) in *Chlorella sorokiniana*. Plant Mol Biol, submitted (1991).

10. Cock JM, Roof LL, Bascomb NF, Gehrke CW, Kuo KC, Schmidt RR: Restriction enzyme analysis and cloning of high molecular weight genomic DNA isolated from *Chlorella sorokiniana* (Chlorophyta). J Phycol 26:361-367 (1990).

11. Dennis ES, Sachs MM, Gerlach WL, Finnegan EJ, Peacock WJ: Molecular analysis of the alcohol dehydrogenase 2 (Adh 2) gene of maize. Nucleic Acids Res 13:727-743 (1985).

12. ~~Feinberg AP, Vogelstein B: A technique for radiolabeling DNA restriction fragments to high specific activity (Addendum). Anal Biochem 137:266-267 (1985).~~

13. Fink GR: Pseudogenes in yeast? Cell 49:5-6 (1987).

14. Gebhardt C, Oliver JE, Forde BG, Saarelainen R, Miflin BJ: Primary structure and differential expression of glutamine synthetase genes in nodules, roots and leaves of *Phaseolus vulgaris*. EMBO J 5:1429-1435 (1986).

15. Hanahan D: Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166:557-580 (1983).

16. Hanley BA, Schuler MA: Plant intron sequences: evidence for distinct groups of exons. Nucleic Acids Res 16:7159-7176 (1988).

17. Hawkins JD: A survey of intron and exon lengths. Nucleic Acids Res 16:9893-9908 (1988).

18. Jacob M, Gallinaro H: The 5′ splice site: phylogenetic evolution and variable geometry of association with U1 RNA. Nucleic Acids Res 17:2159-2180 (1989).

19. Joshi CP: Putative polyadenylation signals in nuclear genes of higher plants: a compilation and analysis. Nucleic Acids Res 15:9627-9640 (1987).

20. Katinakis P, Verma DPS: Nodulin-24 gene of soybean codes for a peptide of the peribacteroid membrane and was generated by tandem duplication of a sequence resembling an insertion element. Proc Natl Acad Sci USA 82:4157-4161 (1985).

21. Kaufer NF, Fried HM, Schwindinger WF, Jasin M, Warner JR: Cycloheximide resistance in yeast: the gene and its protein. Nucleic Acids Res 11:3123-3135 (1983).

22. Kinnaird JH, Fincham JRS: The complete nucleotide sequence of the *Neurospora crassa am* (NADP-specific glutamate dehydrogenase) gene. Gene 26:253-260 (1983).

23. Mattaj IW, McPherson MJ, Wootton JC: Localization of a strongly conserved section of coding sequence in glutamate dehydrogenase genes. FEBS Lett 147:21-25 (1982).

24. McPherson MJ, Wootton JC: Complete nucleotide sequence of the *Escherichia coli gdhA*. Nucleic Acids Res 11:5257-5266 (1983).

25. ~~Meredith MJ, Gronostajski RM, Schmidt RR: Physical and kinetic properties of the nicotinamide-adenine-dinucleotide-specific glutamate dehydrogenase purified from *Chlorella sorokiniana*. Plant Physiol 61:967-974 (1978).~~

26. Mount SM: A catalogue of splice junction sequences. Nucleic Acids Res. 10:459-472 (1982).

27. Nadal-Ginard B, Gallego ME, Andreadis A: Alternative splicing: mechanistic and biological implications of generating multiple proteins from a single gene. In : Setlow JE (ed) Genetic Engineering. Vol. 9. Plenum Press, New York (1987).

28. Proudfoot NJ, Brownlee GG: 3′ non-coding region sequences in eukaryotic messenger RNA. Nature 263:211-214 (1976).

29. Prunkard DE, Bascomb NF, Molin WT, Schmidt RR: Effect of different carbon sources on the ammonium induction of different forms of NADP-specific glutamate dehydrogenase in *C. sorokiniana* cells cultured in light and dark. Plant Physiol 81:413-422 (1986).

30. Prunkard DE, Bascomb NF, Robinson RW, Schmidt RR: Evidence for chloroplastic localization of ammonium-inducible glutamate dehydrogenase and synthesis of its subunits from a cytosolic precursor-protein in *Chlorella sorokiniana*. Plant Physiol 81:349-355 (1986).

31. Reed KC, Mann DA: Rapid transfer of DNA from agarose gels to nylon membranes. Nucl Acids Res 13:7207-7221 (1985).

32. Sanger F, Miklen S, Coulson AR: DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 74:5463-5467 (1977).

33. Sauer N, Tanner W: The hexose carrier from *Chlorella*: cDNA cloning of a eucaryotic H^+-cotransporter. FEBS Lett 259:43-46 (1989).

34. Southern E: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503-517 (1975).

35. Tischer E, DasSarma S, Goodman HM: Nucleotide sequence of an alfalfa glutamine synthetase gene. Mol Gen Genet. 203:221-229 (1986).

36. Turner KJ, Bascomb NF, Lynch JJ, Molin WT, Thurston CF, Schmidt RR: Evidence for messenger ribonucleic acid of an ammonium-inducible glutamate

dehydrogenase and synthesis, covalent modification, and degradation of enzyme subunits in uninduced *Chlorella sorokiniana* cells. J Bacteriol 146:578-589 (1981).

37. Turner KJ, Gronostajski RM, Schmidt RR: Regulation of initial rate of induction of nicotinamide-adenine-dinucleotide-phosphate-specific glutamate dehydrogenase during the cell cycle of synchronous *Chlorella*. J Bacteriol 134:1013-1019 (1978).

38. Valle F, Becerril B, Chen E, Seeburg P, Heyneker H, Bolivar F: Complete nucleotide sequence of the glutamate dehydrogenase gene from *Escherichia coli* K-12. Gene 27:193-199 (1984).

39. Werneke JM, Chatfield JM, Ogren WL: Alternative mRNA splicing generates the two ribulosebisphosphate carboxylase/oxygenase activase polypeptides in spinach and *Arabidopsis*. The Plant Cell 1:815-825 (1989).

40. Wootton JC: The coenzyme-binding domains of glutamate dehydrogenases. Nature 252:542-546 (1974).

41. Yanisch-Perron C, Vieira J, Messing J: Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mp18 and pUC19 vectors. Gene 33:103-119 (1985).

42. Yeung AT, Turner KJ, Bascomb NF, Schmidt RR: Purification of an ammonium-inducible glutamate dehydrogenase and the use of its antigen affinity column-purified antibody in specific immunoprecipitation and immunoadsorption procedures. Anal Biochem 110:216-228 (1981).

43. Yoshimatsu T, Nagawa F: Control of gene expression by artificial introns in *Saccharomyces cerevisiae*. Science 244:1346-1348 (1989).

Figure Legends

Fig. 1. Restriction maps and exon domains of *C. sorokiniana* genomic DNA clones, ~~pGDg 14.10.1, 8.4.4, 14.4.1, and 15.2.1. The entire coding region of the *C. sorokiniana* consensus cDNA described by Cock *et al.* [9] was used to determine the position of 22~~ exons (black boxes) which are interrupted by introns with nuclear consensus splice sites at the exon/intron junctions. The heavy black-lines indicated on the restriction maps, for pGDg 14.10.1 and 8.4.4, correspond to regions which have been sequenced in both directions. The restriction maps were generated using *Acc*I (Ac), *Bam*H1 (B), *Bgl*II (Bg), *Eco*RI (R), *HindIII* (H), *Kpn*I (K), *Sma*I (Sm), *Xba*I (Xb), and *Xho*I (Xh).

Fig. 2. Comparison of the size and number of *Pvu*II restriction fragments derived from *C. sorokiniana* genomic DNA and four putative NADP-GDH genomic clones hybridized to a *C. sorokiniana* cDNA probe (pGDc7; ref. 9) and washed under low stringency (A) or high stringency (B). The low and high stringency washes were: 2 x SSC at room temperature and 0.1 x SSC at 65°C, respectively. Lanes 1,2,3,4 and CG contained digests from genomic clones (see Fig. 1) 8.4.4, 14.4.1, 14.10.1, and 15.2.1, and total genomic DNA, respectively.

Fig. 3. Nucleotide sequence of a *C. sorokiniana* NADP-GDH gene which contains at least 22 exons. The exons span 7143 bp within a 9872 bp region of the genome that was sequenced. The entire NADP-GDH consensus cDNA sequence, described by Cock *et al* [9], resides within the 22 exons. The positions of the exons in this figure are identified by their corresponding deduced amino acid sequences (universal one-letter amino acid code). The highly conserved region identified [9] in the consensus cDNA is distributed over six exons encompassing 2.09 kb as indicated by the positions of the two arrows. The separate arrow head shows the position of the polyadenylation site and the asterisk the position of the putative stop codon. The sequence corresponding to the 3′-untranslated region is underlined.

Fig. 4. Southern blot analysis of restriction fragments obtained by digestion of *C. sorokiniana* genomic DNA with *Ava*II (Av), *Pst*I (P), *Pvu*II and *Pst*I (Pv:P), *Taq*I (T), and *Pvu*II (Pv). A and B, the blots were hybridized to the 378 bp probe from the 3′-untranslated region and to the 242 bp probe from the highly conserved region of the NADP-GDH cDNA [9], respectively. The stringency of the washes was 0.1 x SSC at 65°C.

Fig. 5. The predicted number and sizes of restriction fragments produced by digestion of the NADP-GDH gene (Fig. 3) with *Pvu*II, *Pst*I, *Taq*I, or *Ava*II which should hybridize with the 242 bp conserved region or the 378 bp 3′-untranslated region probes [9]. The black portion of the fragments shows the region of hybridization within the fragments produced by complete digestion of the restriction sites indicated by the arrows. The dashed-lines show the sizes of restriction fragments resulting from incomplete digestion or by possible 5′-methylcytosine inhibition at certain cleavage sites.

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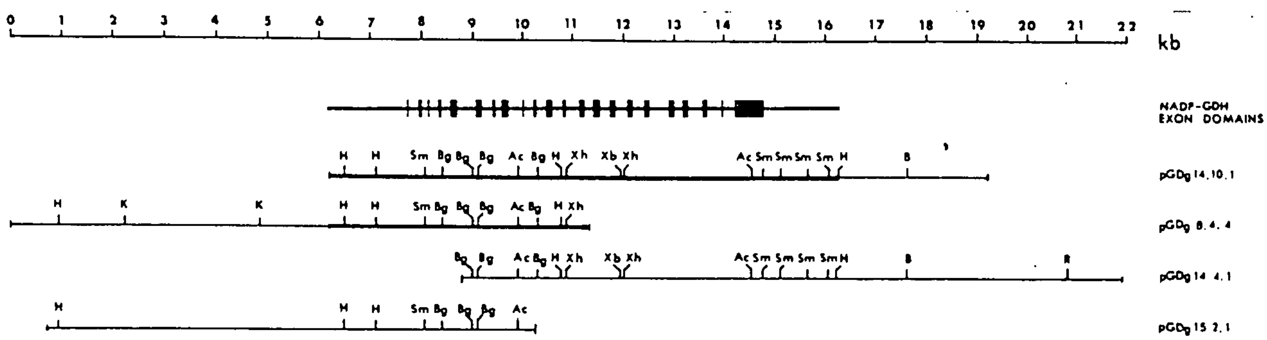
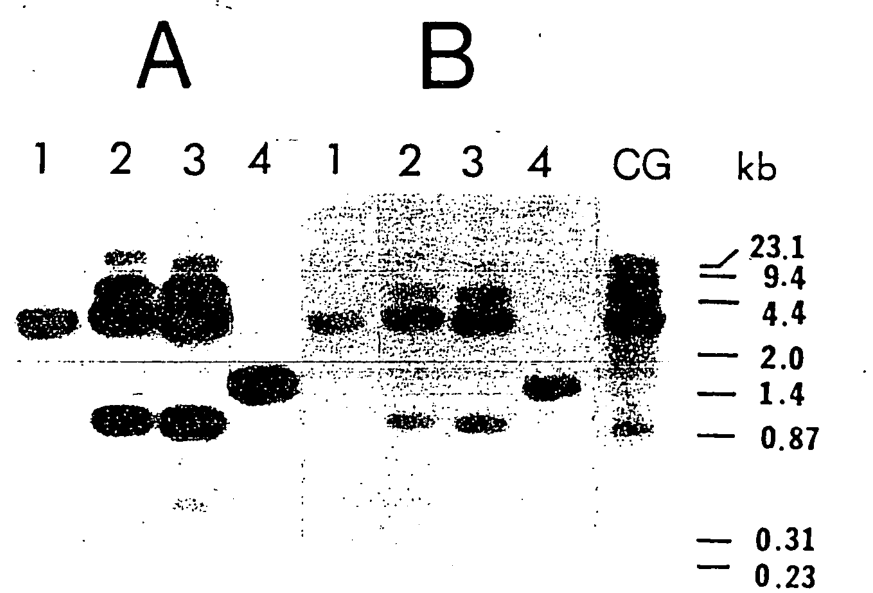
Fig. 6. Southern blot analysis of restriction fragments obtained by digestion of the *C. sorokiniana* genomic DNA with *Sma*I or *Taq*I and hybridized with either the 242 bp conserved region probe (A) or the 378 bp 3′-untranslated region probe (B) and washed ~~under high stringency conditions. The arrow shows the position in the gel of undigested genomic DNA.~~

Cock et al. Fig. 1

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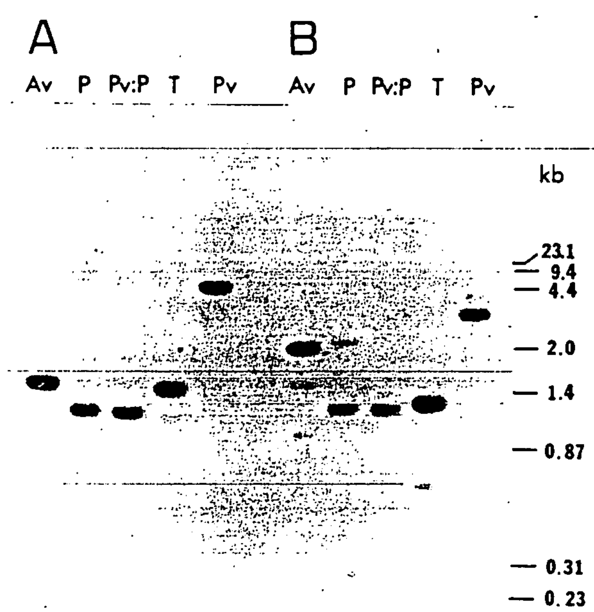
Cock et al. Fig. 4



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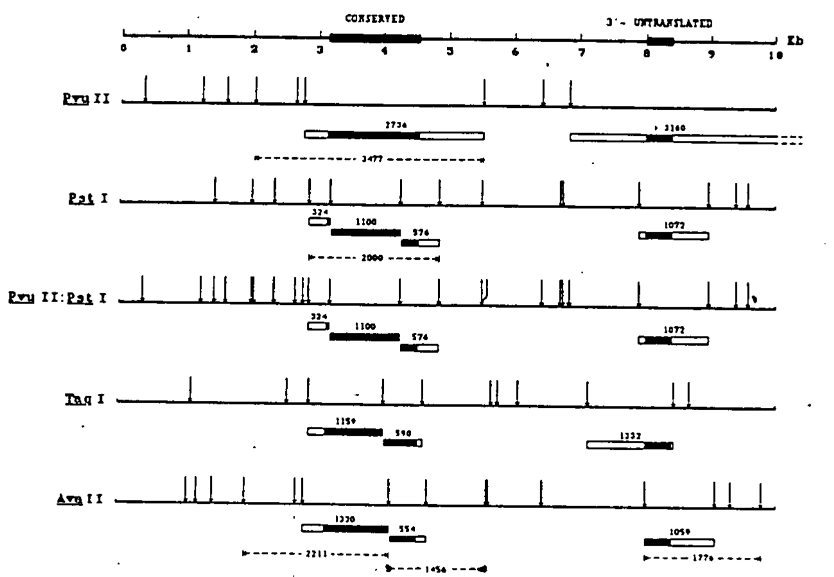
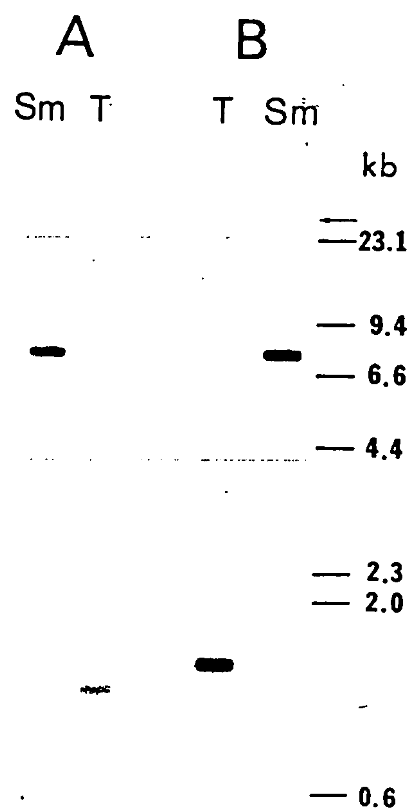
Cock et al. Fig. 5

HYBRIDIZATION REGIONS OF PROBES

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OMB No.3145-0060
NSF Form 1 (5/90)

PROPOSAL EVALUATION FORM

POSAL NO.	INSTITUTION	PLEASE RETURN BY
CB-9103495	U of Florida	04/01/91

RINCIPAL INVESTIGATOR	NSF PROGRAM
Robert R. Schmidt	CELLULAR BIOCHEMISTRY PRO

TITLE Development of Transgenic C3 Plants Requiring Less ATP for ~~NH4+ Assimilation~~

Please evaluate this proposal using the criteria presented on the back of this review form. Continue on additional sheet(s) as necessary.

The overall goal of this research is to replace the gene(s) for glutamine synthetase/GOGAT, the principal system for ammonia assimilation in higher plants, with the gene for the high NH_4^+ affinity isozyme of glutamate dehydrogenase. The P.I. has had considerable experience with this latter gene/enzyme system and the proposed research will certainly break new ground which could lead to more "energy efficient" plants. At this stage, the proposed research could be classified as high risk but the potential payoff in terms of development of plant biotechnology could be considerable. One could question the basic premise of the research - ie., that plant productivity and growth efficiency could be enhanced by the replacement of GS/GOGAT with GDH but its the kind of question that can't be answered until the work has been done. In light of the P.I.'s previous record and background, the probability of useful information and advances in our knowledge of plant molecular biology as it relates to the important process of nitrogen assimilation coming from the proposed research is quite good. It will also serve as a fertile training environment for future plant molecular biologists. I would therefore recommend funding with a high priority (very good/excellent) but for a three year period (rather than 4 years) to allow early evaluation of research progress. The proposed budget appears to be appropriate and well justified.

OVERALL RATING: ☒ EXCELLENT ☒ VERY GOOD ☐ GOOD ☐ FAIR ☐ POOR

don't think that trying to determine if that has an impact on energy required for photorespiration is one of them. It may also be very worthwhile to try to express Chlorella GDH in E. coli in order to produce large amounts so that the enzyme could be crystallized and its structure compared to other GDH forms to try to evaluate a reason for the Chlorella GDH's low Km for ammonium relative to the high Km of other GDH's.

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OMB No.3145-0060

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PROPOSAL EVALUATION FORM

POSAL NO.	INSTITUTION		PLEASE RETURN BY
CB-9103495	U of Florida		03/08/91
PRINCIPAL INVESTIGATOR Robert R. Schmidt		NSF PROGRAM CELLULAR BIOCHEMISTRY PRO	

TITLE Development of Transgenic C3 Plants Requiring Less ATP for NH_4^+ Assimilation

Please evaluate this proposal using the criteria presented on the back of this review form. Continue on additional sheet(s) as necessary.

The PI proposes to isolate an Arabidopsis mutant lacking the GS/GOGAT system for ammonia assimilation and transform the mutant with a gene from Chlorella that encodes a chloroplastic glutamate dehydrogenase. The PI proposes to conduct transformation with several different constructs that have: 1) various promoters, including the native Chlorella promoter, 2) genomic clones containing introns or cDNA clones with introns removed, 3) various transit peptides, including the native Chlorella transit peptide.

This is a rather novel proposal that has some attractive features to it, but also has some potential problems. The basic idea is an interesting one, i.e., replace one ammonia assimilation pathway with a theoretically more efficient one and see if the plant profits from this new pathway. One potential problem is that the Arabidopsis mutant lacking GS has not yet been produced. It should be possible to generate such a mutant (one has been isolated in barley) and the proposed scheme seems reasonable. However, the screen conducted by Somerville was quite exhaustive and there may be some reason that a GS mutant was not found among the many different mutants that show differential growth at high CO_2. Because this is a critical step and the entire project is stalled until the mutant can be isolated it would have been reassuring if it were already available.

By trying all the different constructs, the project should yield some interesting insights into the practical aspects of getting a foreign gene expressed and getting the gene product transported into chloroplasts. However, it is also a problem in that it greatly increases the number of different transformations that need to be conducted and the work load in analyzing all these different transgenic plants. Thus, I would suggest the PI should prioritize the different transformations that will be conducted and analyzed. Despite these reservations, I think the overall proposal is a novel one and worthy of serious consideration for funding.

OVERALL RATING: ☐ EXCELLENT ☒ VERY GOOD ☐ GOOD ☐ FAIR ☐ POOR

don't think that trying to determine if that has an impact on energy required for photorespiration is one of them. It may also be very worthwhile to try to express Chlorella GDH in E. coli in order to produce large amounts so that the enzyme could be crystallized and its structure compared to other GDH forms to try to evaluate a reason for the Chlorella GDH's low Km for ammonium relative to the high Km of other GDH's.

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OMB No.3145-0060

PROPOSAL EVALUATION FORM

OSAL NO.	INSTITUTION	PLEASE RETURN BY
CB-9103495	U of Florida.	04/01/91

RINCIPAL INVESTIGATOR	NSF PROGRAM
Robert R. Schmidt	CELLULAR BIOCHEMISTRY PRO

TITLE Development of Transgenic C3 Plants Requiring Less ATP for NH4+ Assimilation

Please evaluate this proposal using the criteria presented on the back of this review form. Continue on additional sheet(s) as necessary.

The basic premise of this proposal is sound. The PI intends to improve the efficiency of ammonium assimilation in Arabidopsis by replacing the GS/GOGAT pathway with an NADP-GDH gene from Chlorella. This metabolic change will save one ATP per ammonium molecule assimilated and may improve the overall energy efficiency of the plant. This is a proposal where the benefits of success exceed the risks involved.

One problem is whether the Chlorella gene will be expressed in Arabidopsis and whether the peptide will be imported into the chloroplast of Arabidopsis. These are the most risky features of this proposal. However, the PI is aware of these problems and intends to examine them fully.

The double mutant described in this proposal may have other mutations that will make any analysis with the mutant difficult. According to the strategy described, a GOGAT mutant obtained from Dr. Sommerville (MSU 254) will be crossed with a GS mutant prepared in the same manner in the PI's laboratory. Both mutants were or will be obtained by EMS mutagenesis. The resulting progeny will contain DNA that has been exposed to two rounds of EMS mutagenesis. The PI should be certain that these plants are as near normal as possible except for the GS/GOGAT phenotype.

It will also be difficult to compare the energy efficiency of the double mutant to the wild-type and the GOGAT mutant since these are not isogenic and may not even be near-isogenic. A number of physiological studies would be necessary to show that the plants differ only in ammonium assimilation. The PI does not mention such experiments.

Despite these problems, I am very interested in the results. This is an intriguing idea that may not tell us the answer in the first three years of funding, but I believe that the PI is capable of making significant progress during the granting period. I encourage funding of this proposal.

OVERALL RATING: ☐ EXCELLENT ☒ VERY GOOD ☐ GOOD ☐ FAIR ☐ POOR

don't think that trying to determine if that has an impact on energy required for photorespiration is one of them. It may also be very worthwhile to try to express Chlorella GDH in E. coli in order to produce large amounts so that the enzyme could be crystallized and its structure compared to other GDH forms to try to evaluate a reason for the Chlorella GDH's low Km for ammonium relative to the high Km of other GDH's.

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OMB No.3145-0060

PROPOSAL EVALUATION FORM

POSAL NO.	INSTITUTION	PLEASE RETURN BY
,CB-9103495	U of Florida	03/08/91

PRINCIPAL INVESTIGATOR	NSF PROGRAM
Robert R. Schmidt	CELLULAR BIOCHEMISTRY PRO

TITLE Development of Transgenic C3 Plants Requiring Less ATP for NH4+ Assimilation

Please evaluate this proposal using the criteria presented on the back of this review form. Continue on additional sheet(s) as necessary.

OVERALL RATING: ☐ EXCELLENT ☐ VERY GOOD GOOD ☒ FAIR ☐ POOR

don't think that trying to determine if that has an impact on energy required for photorespiration is one of them. It may also be very worthwhile to try to express Chlorella GDH in E. coli in order to produce large amounts so that the enzyme could be crystallized and its structure compared to other GDH forms to try to evaluate a reason for the Chlorella GDH's low Km for ammonium relative to the high Km of other GDH's.

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Schmidt Proposal No. DCB-9103495

This proposal begins with the premise that C-3 plants expend a large amount of energy (both ATP and reducing power) refixing ammonium released in photorespiration. Is this really true? I doubt that the amount of energy expended refixing ammonium released in photorespiration relative to the total utilized during the life of a leaf is very large. If one considers that "physiological quantum requirements" for CO_2 fixation in C-3 plants (approximately 21 photons/mol carbon, measured by McCree, 1972, for 18 species) ~~are already greater than the amount calculated to be consumed by carbon and ammonium fixation in photorespiring leaves (18 quanta/1~~ $\cancel{CO_2 + 1\ NH_4^+}$ ~~for a C-3 plant calculated by various investigators from biochemical pathways and assumptions about the amount of carbon moving into the C-2 oxidation~~ pathway), it seems unlikely that reducing the amount of ATP required for ammonium refixation by 1 ATP/NH_4^+ would have much impact on overall growth of a whole plant. At best, it appears that this might reduce the ~~energy~~ requirement by 5%, but if the plant can generate excess ATP (as many investigators feel it can), one would be hard pressed to find a method to evaluate this small of a change in the energy requirement for growth of whole plants. This is especially true if one considers the variation in plant respiration due to temperature changes, which would indicate that plants do not and cannot maintain a very tight relationship between growth and availability of ATP. However, it is clear that if plants are grown in sufficiently high concentrations of CO_2, such that photorespiration is completely repressed, yields are greater (for example, in experiments done with soybeans at DuPont in the 1970's where bean yield was shown to increase by 20 to 30% in high CO_2). However, completely repressing photorespiration virtually halves the energy required for fixing 1 CO_2, when calculated by analysis of biochemical pathways. Hence, I challenge the basic premise of this proposal: is the hypothesis that reducing the amount of energy required for refixation of ammonium generated in photorespiration by 1 ATP would have an impact on plant growth, testable? I seriously doubt it!

The PI's idea that this can be tested by generating mutants of Arabidopsis which are deficient in both GOGAT and chloroplastic GS, followed by transformation with Chlorella chloroplastic GDH and studies of the transgenic plants to determine if they grow better than wild-type plants with normal GS/GOGAT pathway, has many barriers to overcome before the basic hypothesis can be tested. First, double mutants in both GS and GOGAT must be generated. This seems possible since Peter Lea did it with barley. However, Somerville did not find these double mutants in his extensive studies of Arabidopsis photorespiratory mutants. Second, it is not clear if the Chlorella GDH will act the same way in the higher plant chloroplast as it does in the algal organelle. The PI describes some of the necessary experiments to determine if the Chlorella GDH can even be targeted to the Arabidopsis chloroplast, which may involve extensive modification of the GDH gene. However, it does seem possible to carry out these modifications and get the enzyme to go into the chloroplast of the transgenic plants (if they can be generated). Third, very exacting studies of plant growth and production must be carried out to determine if the presence of chloroplastic GDH will have any impact. It is not at all clear that chloroplastic GDH would allow the plants to grow under normal air conditions (since the mutants would require a high CO_2 atmosphere for growth).

Overall, there are many uncertainties surrounding the proposed study. The PI has very little experience with a number of the experimental protocols that would be required. The prognosis for gaining significant insight into how to make more efficient plants with reduced energy requirement for photorespiration is very poor. I suggest that the PI first propose to work out how to get Chlorella GDH into the chloroplast of any plant (ie. make transgenic plants with this enzyme targeted to their chloroplasts) and determine if a GDH with properties like those found in Chlorella can be isolated and characterized from the transgenic plants. Thus, I would suggest turning around the objectives of the proposal and showing that Chlorella GDH will really function in higher plants, before attempting to go after Arabidopsis double mutants. My guess is that if he can demonstrate functionality of Chlorella GDH in higher plant chloroplasts, that some one with more experience with generating mutants of Arabidopsis could be attracted as a collaborator to help out with making the double mutant necessary to test this far-fetched hypothesis. Let me make it clear that I think there are some worthwhile reasons to try to express the Chlorella GDH in a higher plant chloroplast, but I don't think that trying to determine if that has an impact on energy required for photorespiration is one of them. It may also be very worthwhile to try to express Chlorella GDH in E. coli in order to produce large amounts so that the enzyme could be crystallized and its structure compared to other GDH forms to try to evaluate a reason for the Chlorella GDH's low Km for ammonium relative to the high Km of other GDH's.

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UNIVERSITY OF FLORIDA

SEND NOTICE OF AWARD TO:
The University of Florida
Division of Sponsored Research
219 Grinter Hall
Gainesville, FL 32611
(904) 392-1582

SPONSORED PROJECTS APPROVAL FORM

AGENCY APPLICATION DEADLINE
(DO NOT LEAVE BLANK)

Date: December 1, 1990

☒ **postmark** ☐ **receipt**

~~University Project #~~ ______ (LEAVE BLANK)

NB

Title of Proposal: Development of Transgenic C_3 Plants Requiring Less ATP for NH_4^+ Assimilation

Submitted to Agency/Program: National Science Foundation
(NOTE TO THE P.I.: Please provide mailing instructions on page 2)

UNIVERSITY ENDORSEMENTS: The attached proposal has been examined by the officials whose signatures appear below. The principal academic review of the proposal is the responsibility of the **Department/Center and College.** If additional space is needed for signatures, please provide them on a separate sheet of paper.

Principal Investigator: (Project Director)

Robert R Schmidt 11-21-90
Date

NAME: Robert R. Schmidt
TITLE: Graduate Research Professor
CAMPUS ADDRESS: 1053 McCarty Hall
TELEPHONE: (904) 392-0237
SOC. SEC. NO. 229-38-8422

Approval by Dean or Director: (If more than one)

N. P. Thompson 11/26/90
Date

NAME: N.P. Thompson
TITLE: Associate Dean for Research

Co-Principal Investigator: (If Applicable)

Date

NAME:
TITLE:
TELEPHONE:
SOC. SEC. NO.

Other Endorsement (If Needed):

Date

NAME:
TITLE:

Department Head:

E. M. Hoffmann 11/21/90
Date

NAME: Edward M. Hoffmann
TITLE: Professor and Chairman

Approval by Vice-President for Agricultural Affairs
(For all projects involving IFAS Personnel)

Carol A. Cook 11/26/90
Date

NAME: Carol A. Cook, Assistant Director
TITLE: IFAS Sponsored Programs

Department Head: (If more than one)

Date

NAME:
TITLE:

Approval by Vice-President for Health Affairs:
(For all projects involving JHMHC Personnel)

Date

NAME:
TITLE:

Approval by Dean or Director:

Date

NAME:
TITLE:

Official Authorized to Sign for the University:
(Leave Blank)

Date

NAME:
TITLE:

Division of Sponsored Research
University of Florida

DSR-1 (6/88)

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TO BE COMPLETED BY PRINCIPAL INVESTIGATOR
(Do Not Complete Shaded Boxes)

PRINCIPAL INVESTIGATOR

Schmidt — Last Name (Print or Type)
R R — Initials
229-38-8422 — Social Security Number

Microbiology & Cell Science — Department or Unit to Administer Account
IFAS/Agriculture — College

CO-PRINCIPAL INVESTIGATOR

Last Name (Print or Type) — Initials — Social Security Number

Department — College

Title of Project: Development of Transgenic C_3 Plants Requiring Less ATP for NH_4^+ Assimilation

INDIRECT COST (IDC):

EIES AND IFAS FACULTY OMIT THIS SECTION.

FOR ALL APPLICABLE IDC ACCRUED, RETURN IDC TO: (CHECK ONE)

_____ (A) PI and Home Department Only.
_____ (B) PI, CO-PI and their Home Departments (equal split).
_____ (C) PI, Center and Home Department.
_____ (D) PI and Center Only (No Department IDC return).
_____ (E) OTHER (provide explanation) _____

NAME OF DEPARTMENT(S) TO ACCRUE IDC, IF APPLICABLE _____

NAME OF CENTER TO ACCRUE IDC, IF APPLICABLE _____

IF SPONSOR DOES NOT ALLOW IDC, PLEASE CHECK HERE: ☐

CERTIFICATIONS AND ASSURANCES

		Exp Date	Number
Human Subjects	☐		
Animal Subjects	☐		
Recombinant DNA/RNA	☒		
FDA Good Lab Practices	☐		
Biohazards	☐		

(If none, please check here): ☐

Proprietary Data	☐
Copyright	☐
Invention	☐
Clearinghouse Review	☐
Classified Research	☐

TYPE:
- New ☒
- Renewal ☐
- Continuation ☐
- Supplemental ☐
- Revised ☐

CATEGORY:
- Research ☒
- Training ☐
- Other ☐

MAILING INSTRUCTIONS:

Mail Original and 18 copies to:

Proposal Processing Unit
Room 223
National Science Foundation
Washington, DC 20550

(Check One)
- ☐ First Class
- ☒ Federal Express
- Samas Acc't. # 621000152
- ☐ Other _____

Send 3 copies to P.I.

DSR USE ONLY

UPN: _____ PROPOSAL DATE: _____ AGENCY CODE: _____

AGENCY: _____

DIVISION: _____

BUDG BEG: _____ BUDG END: _____ PROJ BEG: _____ PROJ END: _____

AMT REQUESTED: _____

INDIRECT COSTS—AMT $: _____ RATE: _____ BASE: _____

OFF CAMPUS— $: _____ RATE: _____ BASE: _____

COST SHARING— AMT $: _____ COST SHARING %: _____

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UFFL: _____ YES _____ NO DATE CONTACTED: _____ NR: _____ INTERNAL: _____ COPIES NEEDED: _____

DSR-1 (6/88)

Instructions for Certification

1. By signing the NSF Proposal Cover Sheet, NSF Form 1207, and submitting this application or grant agreement, the grantee is providing the certifications set out below.

2. The certification set out below is a material representation of fact upon which reliance was placed when the agency determined to award the grant. If it is later determined that the grantee knowingly rendered a false certification, or otherwise violates the requirements of the Drug-Free Workplace Act, the agency, in addition to any other remedies available to the Federal Government, may take action authorized under the Drug-Free Workplace Act.

~~3. For grantees other than individuals, Alternate I applies.~~

~~4. For grantees who are individuals, Alternate II applies.~~

Certification Regarding Drug-Free Workplace Requirements

Alternate I (Grantees Other Than Individuals)

The grantee certifies that it will or will continue to provide a drug-free workplace by:

(a) Publishing a statement notifying employees that the unlawful manufacture, distribution, dispensing, possession or use of a controlled substance is prohibited in the grantee's workplace and specifying the actions that will be taken against employees for violation of such prohibition;

(b) Establishing an ongoing drug-free awareness program to inform employees about—

(1) The dangers of drug abuse in the workplace;

(2) The grantee's policy of maintaining a drug-free workplace;

(3) Any available drug counseling, rehabilitation and employee assistance programs; and

(4) The penalties that may be imposed upon employees for drug abuse violations occurring in the workplace;

(c) Making it a requirement that each employee to be engaged in the performance of the grant be given a copy of the statement required by paragraph (a);

(d) Notifying the employee in the statement required by paragraph (a) that, as a condition of employment under the grant, the employee will—

(1) Abide by the terms of the statement; and

(2) Notify the employer in writing of his or her conviction for a violation of a criminal drug statute occurring in the workplace no later than five calendar days after such conviction;

(e) Notifying the agency in writing, within 10 calendar days after receiving notice under subparagraph (d)(2) from an employee or otherwise receiving actual notice of such conviction.

Employers of convicted employees must provide notice, including position, title, to every grant officer or other designee on whose grant activity the convicted employee was working, unless the Federal agency has designated a central point for the receipt of such notices. Notice shall include the identification number(s) of each affected grant;

(f) Taking one of the following actions, within 30 calendar days of receiving notice under subparagraph (d)(2), with respect to any employee who is so convicted—

(1) Taking appropriate personnel action against such an employee, up to and including termination, consistent with the requirements of the Rehabilitation Act of 1973, as amended; or

(2) Requiring such employee to participate satisfactorily in a drug abuse assistance or rehabilitation program approved for such purposes by a Federal, State, or local health, law enforcement, or other appropriate agency;

(g) Making a good faith effort to continue to maintain a drug-free workplace through implementation of paragraphs (a), (b), (c), (d), (e) and (f).

Alternate II (Grantees Who Are Individuals)

(a) The grantee certifies that, as a condition of the grant, he or she will not engage in the unlawful manufacture, distribution, dispensing, possession or use of a controlled substance in conducting any activity with the grant.

(b) If convicted of a criminal drug offense resulting from a violation occurring during the conduct of any grant activity, he or she will report the conviction, in writing, within 10 calendar days of the conviction, to every grant officer or other designee, unless the Federal agency designates a central point for the receipt of such notices. When notice is made to such a central point, it shall include the identification number(s) of each affected grant.

(For NSF, grantee notification should be made to the Award Management and Oversight Branch, Division of Grants and Contracts, NSF, Washington, DC 20550)

THIS PAGE MUST BE SUBMITTED AS PART OF THE PROPOSAL

This certification is required for an award of a Federal contract, grant, or cooperative agreement exceeding $100,000 and for an award of a Federal loan or a commitment providing for the United States to insure or guarantee a loan exceeding $150,000.

CERTIFICATION REGARDING LOBBYING

Certification for Contracts, Grants, Loans, and Cooperative Agreements

The undersigned* certifies, to the best of his or her knowledge and belief, that:

(1) No Federal appropriated funds have been paid or will be paid, by or on behalf of the undersigned,* to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with the awarding of any federal contract, the making of any Federal grant, the making of any Federal loan, the entering into of any cooperative agreement, and the extension, continuation, renewal, amendment, or modification of any Federal contract, grant, loan, or cooperative agreement.

(2) If any funds other than Federal appropriated funds have been paid or will be paid to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with this Federal contract, grant, loan, or cooperative agreement, the undersigned* shall complete and submit Standard Form-LLL, "Disclosure Form to Report Lobbying," in accordance with its instructions.

(3) The undersigned* shall require that the language of this certification be included in the award documents for all subawards at all tiers (including subcontracts, subgrants, and contracts under grants, loans, and cooperative agreements) and that all subrecipients shall certify and disclose accordingly.

This certification is a material representation of fact upon which reliance was placed when this transaction was made or entered into. Submission of this certification is a prerequisite for making or entering into this transaction imposed by section 1352, title 31, U.S. Code. Any person who fails to file the required certification shall be subject to a civil penalty of not less than $10,000 and not more than $100,000 for each such failure.

Statement for Loan Guarantees and Loan Insurance

The undersigned* states, to the best of his or her knowledge and belief, that:

If any funds have been paid or will be paid to any person for influencing or attempting to influence an officer or employee of any agency, a Member of Congress, an officer or employee of Congress, or an employee of a Member of Congress in connection with this commitment providing for the United States to insure or guarantee a loan, the undersigned* shall complete and submit Standard Form-LLL, "Disclosure Form to Report Lobbying," in accordance with its instructions.

Submission of this statement is a prerequisite for making or entering into this transaction imposed by section 1352, title 31, U.S. Code. Any person who fails to file the required statement shall be subject to a civil penalty of not less than $10,000 and not more than $100,000 for each such failure.

*By signing the Cover Sheet and submitting this page as part of the proposal, the applicant is providing Certification Regarding Lobbying.

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COVER SHEET FOR PROPOSALS TO THE NATIONAL SCIENCE FOUNDATION

APPENDIX IV

FOR CONSIDERATION BY NSF ORGANIZATION UNIT (Indicate the most specific unit known, i.e. program, division, etc.)	PROGRAM ANNOUNCEMENT/SOLICITATION NO./CLOSING DATE
Cellular Biochemistry	

EMPLOYER IDENTIFICATION NUMBER (EIN) or TAXPAYER IDENTIFICATION NUMBER (TIN)	SHOW PREVIOUS AWARD NO. IF THIS IS: ☐ A RENEWAL or ☐ AN ACCOMPLISHMENT-BASED RENEWAL	IS THIS PROPOSAL BEING SUBMITTED TO ANOTHER FEDERAL AGENCY? YES _____ NO XX ; IF YES, LIST ACRONYM(S)
1-596001874-H4		

NAME OF ORGANIZATION TO WHICH AWARD SHOULD BE MADE:	INSTITUTION CODE (if known)
~~University of Florida - IFAS~~	~~0015354000~~

~~ADDRESS OF ORGANIZATION (INCLUDE ZIP CODE) Division of Sponsored Research, 219 Grinter Hall,~~
University of Florida, Gainesville, FL 32611

IS SUBMITTING ORGANIZATION: ☐ For-Profit Organization; ☐ Small Business; ☐ Minority Business; ☐ Woman-Owned Business

BRANCH/CAMPUS/OTHER COMPONENT (Where work is performed, if different)	INSTITUTIONAL CODE (if known)
Institute of Food and Agricultural Sciences	same as above

TITLE OF PROPOSED PROJECT

Development of Transgenic C_3 Plants Requiring Less ATP for NH_4^+ Assimilation

REQUESTED AMOUNT	PROPOSED DURATION (1-60 months)	REQUESTED STARTING DATE
$370,715	48 months	

CHECK APPROPRIATE BOX(ES) IF THIS PROPOSAL INCLUDES ANY OF THE ITEMS LISTED BELOW:

- ☐ Vertebrate Animals
- ☐ Human Subjects
- XX Research Involving Genetically Engineered Organisms
- ☐ Historical Places
- ☐ National Environmental Policy Act
- ☐ Proprietary and Privileged Information
- ☐ International Cooperative Activity: ____________ Country/Countries
- ☐ Facilitation Award for Handicapped
- ☐ Research Opportunity Award
- ☐ Disclosure of Lobbying Activities

PI/PD DEPARTMENT	PI/PD PHONE NUMBER/ELECTRONIC MAIL ADDRESS	PI/PD FAX NUMBER
Microbiology & Cell Science	(904) 392-0237	

NAMES (TYPED)	SOCIAL SECURITY NO.*	HIGHEST DEGREE & YEAR	SIGNATURE
PI/PD Robert R. Schmidt Graduate Research Professor	229-38-8422	Ph.D. 1961	Robert R Schmidt
Co-PI/PD			
Co-PI/PD			
Co-PI/PD			
Co-PI/PD			

By signing and submitting this proposal, the individual applicant or the authorized official of the applicant institution is providing the certifications regarding Federal debt status, debarment and suspension, drug-free workplace, and lobbying activities, as set forth in *Grants for Research and Education in Science and Engineering*, NSF 90-77 (8/90).

(If answering "yes" to either, please provide explanation.)	YES	NO
Is the organization delinquent on any Federal debt?		X
Is the organization or its principals presently debarred, suspended, proposed for debarment, declared ineligible, or voluntarily excluded from covered transactions by any Federal department or agency?		X

AUTHORIZED INSTITUTIONAL REPRESENTATIVE	SIGNATURE	DATE
NAME/TITLE (TYPED)		

TELEPHONE NUMBER	ELECTRONIC MAIL ADDRESS	FAX NUMBER

*Submission of social security numbers is voluntary and will not affect the organization's eligibility for an award. However, they are an integral part of the NSF information system and assist in processing the proposal. SSN solicited under NSF Act of 1950, as amended.

TABLE OF CONTENTS

PROJECT SUMMARY

Under photorespiratory conditions, C_3 plants expend a large amount of ATP/reducing equivalents for net assimilation and reassimilation of NH_4^+ by the chloroplastic glutamine synthetase/glutamate synthase (GS/GOGAT) pathway. Chemical mutagenesis and Agrobacterium-mediated transformation of Arabidopsis will be used to replace its GS/GOGAT pathway with a Chlorella chloroplastic glutamate dehydrogenase (α-NADP-GDH) which has a high NH_4^+ affinity. The cDNA and nuclear gene, encoding the precursor-protein for this enzyme, will be inserted into a binary vector for transformation of root explants which will be regenerated into plants. NADP-GDH cDNA/gene constructs will be placed under the control of homologous or heterologous promoters. Chloroplast transit-peptides from Chlorella and Arabidopsis will be tested for their abilities to direct the import of the precursor-protein into Arabidopsis chloroplasts. Constructs are also designed to determine if the precursor-mRNA transcript from the NADP-GDH gene (with many introns) will be processed correctly. Transgenic plants expressing NADP-GDH activity will be analyzed to ascertain whether an increase in efficiency of NH_4^+ assimilation is translated into a net gain in plant productivity.

PROJECT DESCRIPTION

A. Objectives

1. To use a combination of chemical mutagenesis and Agrobacterium-mediated transformation of Arabidopsis thaliana to replace its chloroplastic glutamine ~~synthetase/glutamate synthase (GS/GOGAT) pathway with the Chlorella sorokiniana chloroplastic NADP-specific glutamate dehydrogenase (NADP-GDH) α-~~ homohexamer which has a very high affinity for NH_4^+.

2. To determine whether this pathway replacement will improve the energy efficiency of NH_4^+ assimilation/reassimilation in a C_3 plant under photorespiratory conditions by saving one ATP for every NH_4^+ incorporated into glutamate.

3. To provide comparative biochemistry/molecular biology data on the ability of gene/cDNA (and associated intron splice-sites, regulatory regions, etc.) and the chloroplast transit peptide sequence from a lower eukaryotic plant cell to be expressed or function in a higher plant.

B. Background, Significance, Progress

In chloroplasts of C_3 higher plants (e.g., Arabidopsis) GS and ferredoxin-dependent GOGAT function together in the biosynthesis of glutamate from NH_4^+ produced (i) by reduction of NO_3^- absorbed from the soil, and (ii) from deamination/decarboxylation of glycine during photorespiration (Fig. 1A). The primary route of inorganic nitrogen into organic nitrogen metabolism in higher plants is via transamination of the α-amino group of glutamate (synthesized by the GS/GOGAT pathway) to α-keto acids (1,2). Ammonium assimilation and reassimilation require a very large expenditure of ATP and reducing equivalents, particularly under photorespiratory conditions (3-5). This ATP expenditure becomes even greater as the nitrogen nutrition of the plant is increased. We have observed (unpublished data) that total GS activity in certain C_4 grasses can increase more than three-fold as the concentration of NO_3^- or NH_4^+ in the nutrient medium is increased from 1 mM to 20 mM.

There appears to be a major difference between some lower organisms and higher plants in how they regulate NH_4^+ assimilation and the utilization of ATP for this process, particularly at high nitrogen levels. At high NH_4^+ levels, many bacteria, fungi, and green algae repress/inhibit the GS/GOGAT pathway and induce a NADP-GDH (6,7) to incorporate NH_4^+ into organic nitrogen metabolism via glutamate (Fig. 1B). By use of this alternate route instead of the GS/GOGAT pathway, one ATP is saved for every glutamate synthesized, thereby making nitrogen assimilation more energy efficient at high nitrogen levels. Most higher plants do not have an NH_4^+ inducible NADP-GDH and therefore assimilate NH_4^+ by the GS/GOGAT pathway regardless of the level of nitrogen nutrition. Although most plants have a constitutive mitochondrial NAD-GDH and some have a chloroplastic NAD(P)-GDH, these GDHs (7) have low affinities for NH_4^+ (K_m of 5-50 mM) compared to the plant GS isoenzymes which have very high affinities for NH_4^+ (K_m of 0.01-0.02 mM). One experimental approach for possibly increasing the efficiency of NH_4^+ assimilation/reassimilation in higher plants is to replace the chloroplastic GS/GOGAT pathway with a high affinity NADP-GDH from a lower

organism.

Research in this laboratory has revealed that the unicellular green alga, Chlorella sorokiniana, has a constitutive, mitochondrial, tetrameric NAD-GDH (identical subunits, each 45,000 Da) and two NH_4^+-inducible chloroplastic, homohexameric NADP-GDH α- and β-isoenzymes (subunits 55,500 or 53,000 Da, respectively) which have strikingly ~~different affinities for NH_4^+ (8-11). By use of a combination of biochemical, immunochemical, and molecular biology procedures, we have obtained evidence (12-14)~~ that a large (7-8 kb) nuclear gene (Fig. 2), containing at least 22 introns, encodes the chloroplastic α- and β-subunits.

When the α- and β-isoenzymes are accumulating together in cells cultured in 29 mM NH_4^+ medium, we can detect only a single-size (2.2 kb) NADP-GDH mRNA on Northern blots, using hybridization probes from the conserved amino-acid coding region or the 3′-untranslated region prepared from a 1.91 kb C. sorokiniana NADP-GDH cDNA (Fig. 3 and 4). From a cDNA library that was prepared from total poly(A)$^+$RNA, isolated from C. sorokiniana cells synthesizing α- and β-isoenzymes, we selected 17 NADP-GDH cDNAs which were restriction mapped and totally or partially sequenced (Fig. 3 B,C). All of these cDNAs have identical nucleotide sequences for the regions that overlap. A 2,146 bp consensus NADP-GDH restriction map is shown in Fig. 3A. Beginning with the second nucleotide from the 5′-terminus of the cDNA consensus nucleotide sequence, an open-reading-frame (ORF) was revealed which ends with a TAA stop-codon at 1,571 bp. This ORF encodes a protein with a molecular weight of 57,401 which is almost the complete size (98%) of the NADP-GDH precursor-protein (58,500 D). This consensus cDNA is missing the 5′-untranslated region, the ATG start-codon, and part of the chloroplast transit-peptide sequence. However, if the α- and β-subunits are encoded by differential processing of a precursor-protein derived from a single mRNA, this cDNA contains the sequences for both mature subunits. A comparison of the deduced amino-acid sequences of the conserved regions, in the NADP-GDH from C. sorokiniana, Escherichia coli, and Neurospora crassa, showed the alga NADP-GDH conserved sequence to be 77% and 73% homologous with the bacterial and fungal sequences, respectively (Fig. 4). The results from Southern blot analyses of digests of C. sorokiniana genomic DNA with various restriction enzymes (e.g., PvuII, TaqI, AvaII, etc.) in which the conserved-region and 3′-untranslated region probes were used, are consistent with there being a single nuclear gene NADP-GDH gene (12-14).

By use of a very specific polysome immunoselection procedure, coupled with oligo(dT) chromatography, we purified the NADP-GDH mRNA 1290-fold to apparent homogeneity from C. sorokiniana cells accumulating primarily the β-homohexamer (35). In vitro translation of this purified mRNA produced a single protein with a molecular weight of 58,500 (35). In vitro translation of total poly(A)$^+$RNA, isolated from cells synthesizing primarily the α- or β-homohexamer resulted in the synthesis of 58,500 Da precursor-protein(s) which were processed in vitro (by C. sorokiniana extracts) to 55,500 Da and 53,000 Da subunits (11,36). These two subunits have very similar peptide maps, and both can be immunoprecipitated by polyclonal antibodies prepared against one of the subunits, indicating that they have a high degree of sequence homology (11).

We have determined (11) that below 3 mM NH_4^+ in the culture medium only the α-homohexamer accumulates in the chloroplast. Above this NH_4^+ concentration, both isoenzymes initially accumulate (i.e., 1st 120 min), then accumulation of the α-subunit

ceases, and only the β-homohexamer continues to accumulate at long induction times (i.e., 3-12 h). From additional nitrogen nutrition studies in which the cells were subjected to rapid transitions between low and high NH_4^+ concentrations, we concluded (11,13) that some type of feedback mechanism switches gene expression from synthesis of the α-subunit to the β-subunit as intracellular nitrogen metabolite(s) reach a certain ~~threshold concentration. Experiments are in progress to determine whether the α/β switch is (i) the differential processing of a single precursor-protein encoded by a single~~ NADP-GDH mRNA, or (ii) specific processing of two slightly different precursor-proteins which are derived from two similar size NADP-GDH mRNAs encoded by the same gene (e.g., two mRNAs produced by alternative splicing of a common precursor-NADP-GDH mRNA). The reason for being tentative, regarding the existence of one or two NADP-GDH mRNAs, is that 10 of the 17 NADP-GDH cDNAs which isolated are missing both their 3'- and 5'-terminal sequences (Fig. 3 B,C). In view of the large number of exons in the NADP-GDH gene (Fig. 2), with some of these exons being as small as 16 bp, it is possible that alternative splicing (16) of the precursor-mRNA could generate two mRNAs of nearly the same size with sequence differences, particularly in the region between the conserved region and the 3'-untranslated region. To determine whether or not all of the NADP-GDH mRNAs have identical 5'-termini, primer extension/anchor PCR (19) is being used with poly(A)$^+$RNA isolated from cells synthesizing either the α- or β-homohexamer. For 3'-terminal analysis, NADP-GDH cDNAs with complete 3'-termini are being isolated and sequenced from new cDNA libraries prepared with poly(A)$^+$RNA from cells synthesizing either the α- or β-homohexamer. These experiments are in progress and will be completed by the time this NSF proposal is funded.

The C. sorokiniana α-homohexamer has a very high affinity for NH_4^+ (M_r ranges between 0.02 mM and 3.5 mM) and is allosteric in that its NH_4^+ K_m varies with NADPH concentration (11). Our search of the scientific literature has not revealed any other reports of a GDH with an NH_4^+ K_m as low as 0.02 mM. This NH_4^+ K_m falls into the range of those reported for higher plant GS isoenzymes (0.01 mM - 0.02 mM). In contrast, the β-homohexamer has a low affinity for NH_4^+ (M_r = 75 mM) and is non-allosteric with respect to NADPH. When cells are synthesizing both α- and β-subunits early during the induction period at high NH_4^+ concentration, homo- and heterohexamers (i.e., 6α, 5α:1β, 4α:2β, 3α:3β, 2α:4β, 1α:5β, and 6β) accumulate within the chloroplast (37). These NADP-GDH heterohexamers presumably have NH_4^+ K_m values which fall between those of the two homohexamers (11). In addition to the process which controls the differential synthesis of the α- and β-subunits, we have shown (20,21) that the levels/activities of the NADP-GDH holoenzymes are regulated by a Ca^{+2} and ATP dependent inactivation/degradation process involving covalent-modification of the subunits as an obligatory step to their degradation (see model, Fig. 5).

It is the α-homohexamer which has potential use in higher plant biotechnology for increasing the energy efficiency of NH_4^+ assimilation in food and biomass crops. Once the molecular mechanism is understood by which the NADP-GDH gene gives rise to α- and β-subunits, the NADP-GDH cDNA and gene will be modified by in vitro mutagenesis so that only the α-isoenzyme can be synthesized in vivo in transgenic plants. The modified cDNA/gene will be used for the biotechnology application discussed below. The unmodified cDNA/gene also will be used in comparative biochemistry/molecular biology studies to determine whether a chloroplast transit-

peptide sequence, introns, etc. from a lower plant will function or be processed, etc. in a higher plant.

C. Experimental Plan and Methods

Selection of Arabidopsis mutant(s) having both low GS activity and absence of GOGAT activity

Arabidopsis (22) and barley (23) mutants have been isolated which are deficient in GOGAT or chloroplastic GS activities, respectively. These mutants were selected for their ability to grow in atmospheres with elevated CO_2 levels (0.8 - 1.0%) but not in normal air. The basis of the mutant selection was that CO_2 competitively inhibits the oxygenase activity of ribulose bisphosphate carboxylase/oxygenase (Rubisco) which catalyzes the formation of phosphoglycollate, the first intermediate on the photorespiratory pathway. Because of the importance of both the chloroplastic GS and GOGAT in the reassimilation of NH_4^+ produced during photorespiration, a deficiency in either of these enzymes leads to accumulation of NH_4^+ in the leaves and rapid inhibition of photosynthesis after these mutants are transferred to air in the light. Under these photorespiratory conditions, the mutants become chlorotic within several days, and can be rescued by returning them to an elevated CO_2 atmosphere in the light. Since plants containing mutations in genes encoding some of the other enzymes in the photorespiratory pathway may also give the chlorotic phenotype under photorespiratory conditions, direct enzyme analysis (22) of leaf extracts is required to identify specific GS and GOGAT mutants.

In leaves of wild-type C_3 plants, such as barley and Arabidopsis, the chloroplastic GS isoenzyme has been shown (24) to represent a much higher percentage of the total GS activity than the cytosolic GS (approx. 85:15, respectively). The higher plant cytosolic and chloroplastic GS isoenzymes are encoded by different nuclear genes (25-28). Wallsgrove et al (23) isolated a barley mutant deficient in the chloroplastic GS but which still contained wild-type levels (i.e., approx. 17% of total GS activity in leaves) of the cytosolic GS. Under elevated CO_2 levels in the light, this barley mutant grew normally, indicating that the remaining wild-type activity of the cytosolic GS was sufficient to meet the glutamine requirement of the plant for biosynthesis of purines, pyrimidines, arginine, histidine, and tryptophan. However, when the mutant plant was placed under photorespiratory conditions, the cytosolic GS by itself was unable to reassimilate the large amount of NH_4^+ produced during photorespiration.

Although an Arabidopsis thaliana (Columbia ecotype) GOGAT mutant (GluS) has been isolated by Somerville and Ogren (22), neither cytosolic nor chloroplastic GS mutants have been isolated yet for this plant. Dr. C. Somerville (Michigan State University) has given us seed of his GOGAT mutant (GluS; MSU 254) for use in this project. However, we will have to isolate Arabidopsis mutants which contain wild-type cytosolic GS activity and are deficient (0-20%) in chloroplastic GS activity. When these chloroplastic GS mutants (homozygous) are isolated, they will be crossed with the GOGAT mutant. From the resulting progeny, a double-mutant homozygous for both the GOGAT and chloroplastic GS mutations will be isolated. This double mutant is required for the development of a transgenic plant in which the biosynthesis of glutamate in the chloroplast will occur via the NADP-GDH α-homohexamer (introduced from Chlorella) instead of the GS/GOGAT pathway (Fig. 1C). Somerville and Ogren (22) observed that, in the Arabidopsis GOGAT mutant under photorespiratory conditions,

the chloroplastic GS rapidly converted free glutamate to glutamine, resulting in the deprivation of free glutamate for use in biosynthesis of the other amino acids. Thus, unless the wild-type level of the chloroplastic GS is low or absent in the transgenic plant, the glutamate synthesized by the NADP-GDH may be rapidly converted to glutamine, resulting in a shrinkage in the pool of available glutamate normally used in transaminase ~~reactions. It should be noted that the primary route for assimilation of inorganic nitrogen into organic nitrogen metabolism is via transamination of the α-amino group~~ of glutamate into the carbon skeletons of amino acid precursors.

We will select Arabidopsis GS mutants by the same procedure described by Somerville and Ogren (22) and Estelle and Somerville (29) to isolate their GOGAT mutant (GluS, MSU 254). Mutagenesis will be accomplished by soaking seeds in a 0.3% solution of ethyl methane sulfonate. This treatment will induce heterozygous mutations in some of the cells which will give rise to the reproductive structure of the plant. This M1 generation will be cultured to maturity in normal air under fluorescent lamps, allowed to self-fertilize, and the seed will be collected. The seed will be germinated at high densities in the light under an atmosphere of 1% CO_2-air, and these M2 progeny will be screened by placing them into normal air for 3-4 days. The plants which show chlorosis will be identified and returned to the high-CO_2 environmental growth chamber and allowed to self-fertilize and produce seed. The seed from each M2 plant then will be germinated separately in the high-CO_2 atmosphere, transferred to normal-air to identify homozygous mutant progeny, and then returned to the high-CO_2 atmosphere for recovery and further growth. After a suitable recovery time from chlorosis, extracts will be prepared from the leaves of these M3 progeny and will be analyzed for total GS activity. When extracts having low total GS activity are identified, these will be further analyzed by ion-exchange chromatography in a Pharmacia FPLC (analytical Mono Q column, NaCl gradient) to determine the ratio of activities of the cytosolic and chloroplastic GS isoenzymes. Those mutants which have a wild-type level of the cytosolic GS, and are deficient (0-20%) the chloroplastic GS, will be allowed to self-fertilize and their seed will be collected. Progeny from these seed will be used in crosses with the GOGAT mutant to produce the chloroplastic GS/GOGAT double-mutants as discussed above.

Agrobacterium-mediated transformation of Arabidopsis

Several types of binary Ti plasmid vectors have been used for the Agrobacterium-mediated transformation of the different ecotypes of Arabidopsis (30-34). In addition to their ability to replicate in both Agrobacterium and E. coli, these binary vectors usually have both left and right border repeats of the T-DNA region, a dominant marker gene (e.g., kanamycin or hygromycin B resistance), several unique restriction sites for insertion of foreign DNA between the T-DNA borders, and an antibiotic gene for maintenance in the resident bacterium (30). For the T-DNA region of the binary vector to be transferred into a plant cell, the Agrobacterium strain must also carry a helper Ti plasmid which provides the necessary transacting vir functions which are lacking in the binary vector. To prevent recombination with binary vectors, helper plasmids have had their T-DNA region deleted.

Binary-vectors carrying the genes for kanamycin or hygromycin B resistance appear to vary in their effectiveness as selectable markers for use in isolation of transformed cells from the various Arabidopsis ecotypes. For example, the Columbia ecotype is

reported (31) to have some natural resistance to kanamycin whereas Wassilskija and Landsberg erecta are very sensitive to this antibiotic. For those ecotypes with some resistance to kanamycin, hygromycin B has proven to be an effective selectable marker. Feldmann and Marks (32) and Feldmann et al. (33) have successfully transformed Arabidopsis (ecotype Wassilskija) by germinating seeds in the presence of Agrobacterium carrying a kanamycin resistant binary vector. Although this transformation method appears to be very simple and convenient, there are reports (personal communication with various scientists) that transformation frequencies are often low and vary among different seed lots. Lloyd et al. (31) have transformed Arabidopsis (Columbia ecotype) by a modified leaf-disk transformation/regeneration method using a binary vector encoding hygromycin B rather than kanamycin. Because uninfected control leaf-tissue of the Columbia ecotype also developed callus in the presence of kanamycin, this antibiotic was ineffective as a selecting agent with this ecotype in the leaf-disk method. However, with the hygromycin-resistant vector, approximately one-third of the original trans-formed leaf pieces survived the hygromycin selection-step and more than 50% of these generated shoots. Four months were required from the time of infection of leaf-tissue pieces with Agrobacterium until the collection of seed from the transformed plants. Valvekens et al. (34) have developed cultural conditions for inducing root-explants, of several Arabidopsis ecotypes, to generate shoots rapidly and at 100% efficiency. By use of this root-explant regeneration procedure, along with a Ti plasmid vector encoding kanamycin resistance, trans-formed seed-producing plants were obtained with an efficiency of 20-80% within 3-months after gene transfer. In addition to a shorter time to obtain seed from transformed tissue, this root explant transformation/regeneration method which employed kanamycin was successful with three different ecotypes (i.e., Columbia, Landberg erecta, and C24). This finding contrasts with the reported difficulties of the Columbia ecotype by the leaf-disk method discussed above (31).

Because the Columbia ecotype was used to select the chloroplastic GOGAT mutant, which was obtained from Dr. Somerville, we initially plan to use the root explant transformation/regeneration method of Valvekens et al. (34) and a Ti plasmid binary vector carrying the kanamycin resistance gene. The binary vector system (GUS Gene Fusion Kit) will be purchased from Clontech Laboratories. This system utilizes A. tumefaciens strain LBA4404 with its helper plasmid based on an octopine Ti plasmid, and several modified binary vectors: plasmid pBI101 (GUS cassette, no promoter), plasmid pBI121 (pBI101 with CaMV 35S promoter, and plasmid BI221 (pBI121 GUS cassette in pUC19). The kit also contains the conjugative plasmid RK2013 in HB101. The aforementioned binary vectors contain, between the right and left borders, the kanamycin resistance gene (npt II) which is driven and terminated by the nopaline synthase (NOS) promoter (NOS-pro) and terminator (NOS-ter), respectively. The β-glucuronidase (GUS) gene in pBI121 is driven and terminated by the CaMV 35S promoter and the NOS-ter, respectively. The 3' and 5' termini of the CaMV 35S promoter and NOS-ter termini, respectively, have unique restriction sites which will permit excision of the GUS gene and its replacement with the Chlorella NADP-GDH cDNA or genomic DNA. To determine if the natural promoter of the Chlorella NADP-GDH gene can be expressed (without or with in vitro mutagenesis) in Arabidopsis, the "promoter-less" GUS cassette in pBI101 will be used. In this binary plasmid, the CaMV 35S promoter has been deleted and a multicloning site has been inserted in its place 5'

to the GUS gene. Thus, various promoters (e.g., NADP-GDH promoter region) can be cloned upstream of GUS which can be used as a reporter gene.

Analysis of expression of Chlorella NADP-GDH cDNA/genomic DNA in transgenic Arabidopsis plants

~~The Arabidopsis GS/GOGAT mutant will be transformed with the aforementioned binary vector(s) carrying a number of different Chlorella NADP-GDH cDNA/genomic~~ DNA constructs:

a. Full-length NADP-GDH cDNA carrying its own ATG start-codon, chloroplast transit-peptide sequence, and its 3'-terminus devoid of its poly(A)tail (i.e., the Nos-ter will provide the terminator/polyadenylation signal).
b. The same cDNA (as a.) modified by replacement of the Chlorella chloroplast transit-peptide sequence with the equivalent higher plant sequence reported (38) for one of the four Arabidopsis Rubisco small subunit precursor-proteins (e.g., standard single-letter code for amino acids for transit-peptide AtB is MASSMLSSAAVVTSPAQATMVAPTGLKSSASFPVTRKANNDITSITSNGGRVSC). Alternatively, we will be screening an Arabidopsis cDNA library with a heterologous GS cDNA probe (Phaseolus vulgaris) to isolate the chloroplastic GS cDNA. In the event that the import and/or processing of precursor-proteins for stromal enzymes, involved in different aspects of chloroplast metabolism (e.g., carbon vs. nitrogen) is/are regulated in part by transit-peptides with sequences differences, it might be advantageous to use the transit-peptide sequence for the Arabidopsis chloroplastic GS instead of the one for the Rubisco small subunit.
c. The same cDNA (as a.) without any chloroplast transit peptide sequence (i.e., the NADP-GDH will be targeted for the cytosol instead of the chloroplast).
d. The entire NADP-GDH genomic DNA clone (gene) containing its natural promoter region, start codon, exons, introns, and termination/polyadenylation signal(s).
e. Another related construct will be the NADP-GDH promoter region by itself.
f. The same NADP-GDH gene (as d.) modified by deletion of its natural promoter region.

The a., b., c., and f. constructs will be inserted into vector pBI121 between CaMV 35S promoter and Nos-ter to replace the deleted GUS gene. The d. construct will be inserted into the "promoter-less" vector pBI101 to replace its GUS gene. The e. construct will be inserted into the "promoter-less" vector pBI101 in front of the GUS gene to determine whether the Chlorella promoter will be able to drive the GUS gene.

Transformants from the root-explants initially will be identified by their kanamycin resistance, and regenerant plants will be allowed to self-fertilize and produce seed in a high-CO_2 lighted, environmental chamber. These seed will be germinated in the presence of kanamycin in a high CO_2 atmosphere and the antibiotic resistant progeny will be identified. These will be placed under photorespiratory conditions (light, normal air) to identify which plants remain green (if any) and those which become chlorotic. The plants which remain green will be allowed to self-fertilize and produce seed under photorespiratory conditions whereas the chlorotic plants will be returned to the high CO_2 atmosphere for seed production. The seed from these plants will be germinated in the low or high CO_2 atmospheres and extracts of their leaves will be analyzed for NADP-GDH activity (spectrophotometrically), NADP-GDH anti-gen (Western blotting), NADP-

GDH mRNA (Northern blotting) and NADP-GDH DNA (Southern blotting). Assays will also be performed for total GS activity to verify that transformation did not alter the wild-type level of the cytosolic GS in the transgenic plant. To confirm that kanamycin resistance is conferred by neomycin phosphotransferase and not by some other mechanism in the transformants, assays for this activity will also be performed. The ~~aforementioned assays for NADP-GDH-antigen, -mRNA, and -DNA will be particularly important for trans-genic plants which become chlorotic under photorespiratory~~ conditions. For example, if the Arabidopsis genome contains the intact NADP-GDH cDNA/gene and the plant does not accumulate active enzyme, it might be possible to identify the biochemical step (i.e., transcription, translation, post-translation) that is limiting the accumulation of active NADP-GDH. If the transgenic plants (green or chlorotic) contain NADP-GDH antigen or activity, their chloroplasts will be isolated and analyzed to ascertain whether the antigen/activity is chloroplast localized. For the plants transformed with NADP-GDH cDNA without a chloroplast transit-peptide sequence, assays will be performed to show whether or not the NADP-GDH is accumulating in the cytosol. From a comparative biochemical/molecular biology viewpoint, the results from the aforementioned assays on the transgenic plants, carrying the different cDNA/gene constructs, are important for identifying possible differences in gene-enzyme regulation (or processing) in higher and lower plants. For example, from the cDNA constructs having the Chlorella or higher-plant chloroplast transit-peptide sequence, it should be possible to show whether the lower plant transit-peptide sequence will direct the NADP-GDH precursor-protein into the Arabidopsis chloroplast and will be recognized and processed by the endopeptidase(s) of this higher plant. Also, another question of comparative biochemistry importance is whether the natural promoter(s) of the Chlorella NADP-GDH gene will be recognized by the regulatory proteins/RNA polymerase of Arabidopsis. Moreover, if the NADP-GDH gene is transcribed into a large precursor mRNA (pre-mRNA) in this higher plant, will the many exons (including one only 16 bp) be spliced together correctly?

Efficiency of inorganic nitrogen assimilation, carbon dioxide fixation, and biomass yield in transgenic Arabidopsis plants expressing chloroplastic NADP-GDH

For every NH_4^+ assimilated into glutamate by the chloroplastic NADP-GDH rather than by the GS/GOGAT pathway, one ATP should be saved. A question of importance from an agricultural biotechnology standpoint is whether this savings in ATP can be translated into a net gain in energy that can be used for anabolic processes by the plant.

Before plant productivity studies are considered, several basic measurements need to be performed on the different (isolates) Arabidopsis transgenic plants having chloroplastic NADP-GDH activity. Due to variations in the number of copies (gene dosage) of the NADP-GDH cDNA/gene that can be inserted into the Arabidopsis genome and in their position(s) in the genome (i.e., adjacent genes/promoters can influence expression of inserted gene), different amounts of NADP-GDH activity may accumulate in the leaves. Firstly, it will be important to rank the transformants on the basis of their amount of leaf NADP-GDH activity. Their degree of resistance to chlorosis under photorespiratory conditions may prove to be correlated to the amount of NADP-GDH activity in their leaves. Secondly, the NH_4^+ concentration in the leaves will be measured before transfer to photorespiratory conditions and during a time-course thereafter. Thirdly, the photosynthetic rate will be measured (22) as a function of time

after transfer to photorespiratory conditions. As controls, the same measurements will be performed on the wild-type and GS/GOGAT mutant (not transformed) Arabidopsis plants.

Because of the possible variation in gene dosage, there could be a wide range of NADP-GDH activities in the transformants. From a plant energy-economy standpoint, ~~the ideal transgenic plants, selected for biomass production measurements, will be those with the lowest levels of NADP-GDH activity which can maintain wild-type levels (or~~ lower) of NH_4^+ in the leaves under photorespiratory conditions. Because the NADP-GDH cDNA/gene insertions into the Arabidopsis genome might lower the activity of some essential plant enzyme unrelated to nitrogen metabolism, a number of NADP-GDH transformants will be evaluated in the biomass productivity studies. The following comparisons will be made between wild-type Arabidopsis and the aforementioned final-selection of transformants during growth (in a random-block design) under photorespiratory conditions:

a. Generation time from seed germination until seed set.
b. Total protein, total RNA, total DNA, lipid, starch, and chlorophyll content of leaves (per fresh and dry weight) at periodic intervals during growth/maturation cycle.
c. Total weight of seed produced.
d. Rates of uptake of NO_3^- and NH_4^+ in separate nutrition experiments vs. developmental stage.
e. Rate of $^{14}CO_2$ incorporation by the intact plant.
f. Leaf ADP/ATP ratio.

D. Figure Legends and Figures

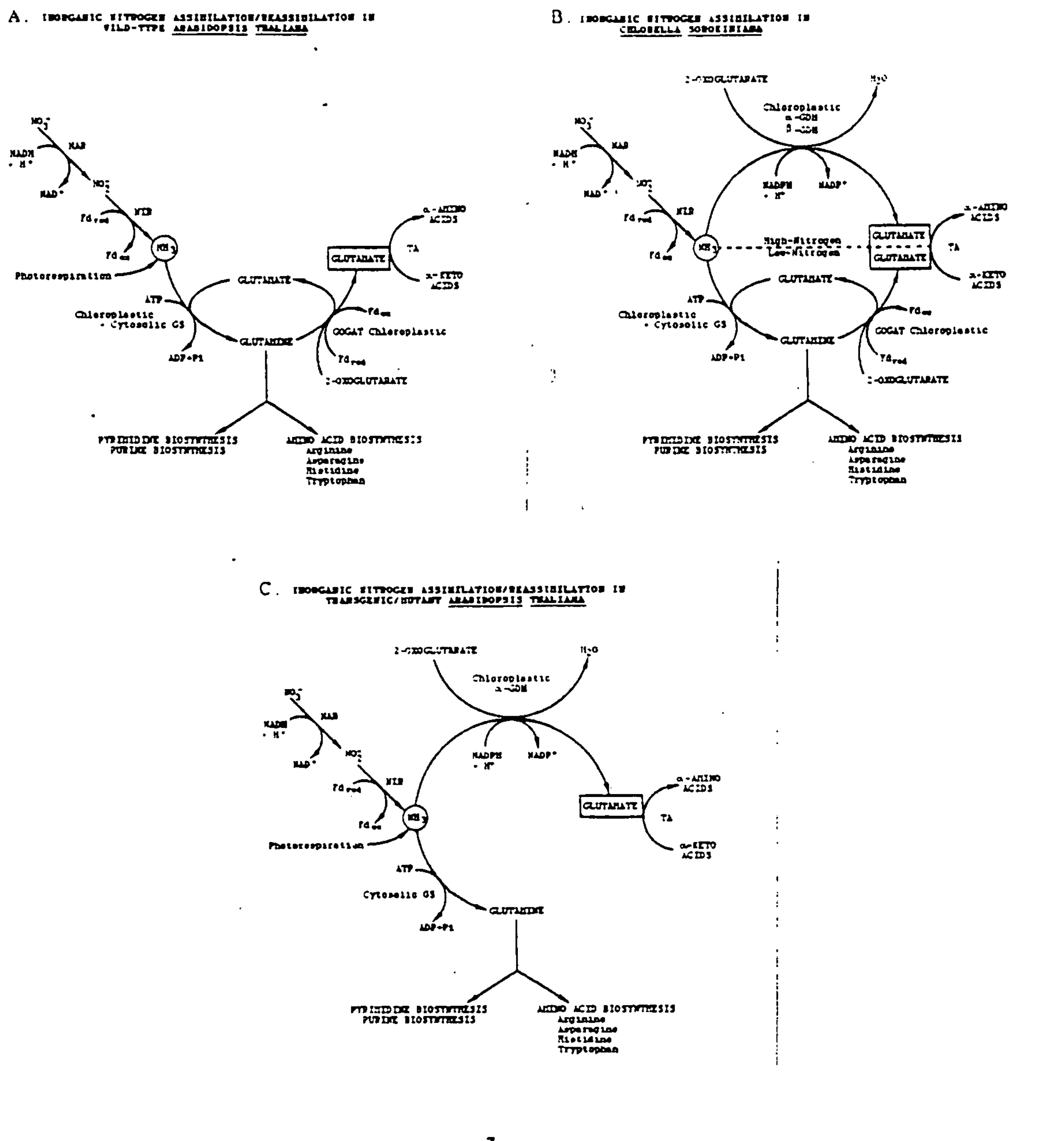
Fig. 1 A,B,C. Pathways of inorganic nitrogen assimilation/reassimilation in A. thaliana leaves, C. sorokiniana cells, and transgenic/mutant A. thaliana leaves.

Fig. 2. Restriction maps and exon domains of NADP-GDH genomic DNA clones, pGDg 14.10.1, 8.4.4, 14.4.1, 15.2.1. The entire region of the NADP-GDH consensus cDNA shown in Fig. 3A was used to determine the positions of 22 exons (black boxes) which are interrupted by introns with nuclear consensus splice sites at the exon/intron junctions. The heavy black-lines indicate those regions in the clones which have been sequenced (14).

Fig. 3 A,B,C. Restriction maps of 17 NADP-GDH cDNAs isolated from a cDNA library prepared from total poly(A)$^+$RNA extracted from cells induced for 80 min in 29 mM NH_4^+ medium. Panel A, 2,146 bp consensus NADP-GDH cDNA restriction map. The heavy and light black-lines are the amino-acid coding region and the 3′-untranslated region, respectively. The regions corresponding to the conserved region probe (242 bp PstI fragment) and the 3′-untranslated region probe (378 bp NarI/SmaI fragment) are indicated. Panel B, the cDNA clones pGDc 2, 3, 6, 7, 10, and 23 were isolated using a heterologous 1.2 kb probe from the gdhA gene from S. thyphimurium. Both strands of pGDc23 (1.91 kb) have been sequenced as indicated by the arrows. Panel C, the cDNA clones, pGDc 30, 31, 32, 33, 34, 35, 36, 38, 39, 42 and 44 were isolated using a homologous 115 bp PstI fragment from near the 5′-end (overlapping into conserved region) of pGDc23 (15).

Fig. 4. Nucleotide sequence of the highly conserved region of a NADP-GDH cDNA, pGDc23, and comparison of its deduced amino acid with the amino acid sequences of Escherichia coli and Neurospora crassa NADP-GDHs (15).

Fig. 5. Diagram of modified model for regulation of activity, synthesis, inactivation, and ~~degradation of α- and β-isoenzymes of NADP-GDH in C. sorokiniana (see ref. 20 for details of original model)~~



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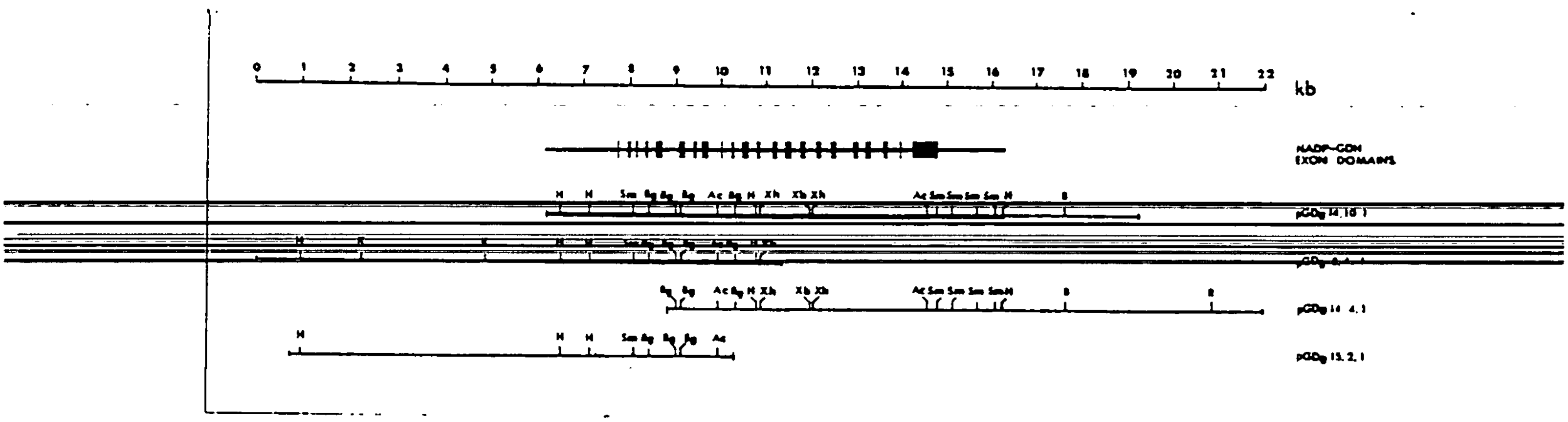

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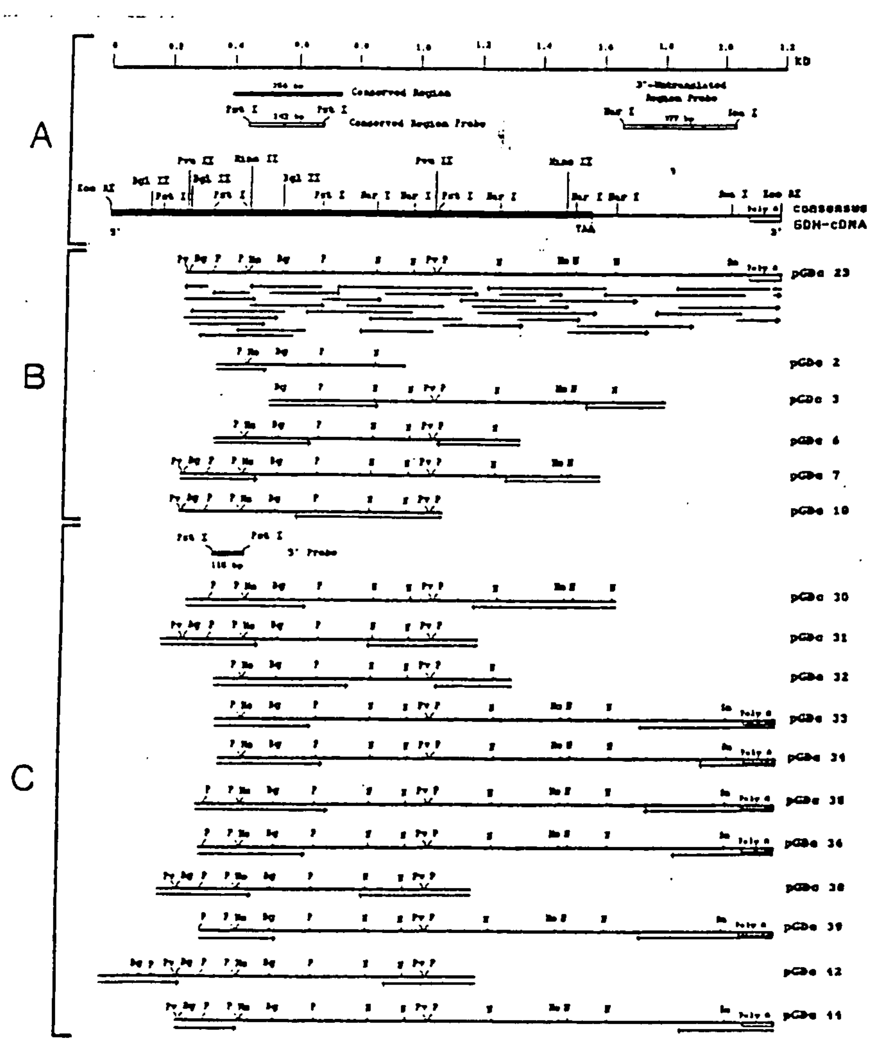

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Nucleotide Sequence of Highly Conserved Region of <u>Chlorella sorokiniana</u> NADP-GDH cDNA and Comparison of Its Deduced Amino Acid Sequence with Amino Acid Sequences from <u>Escherichia coli</u> and <u>Neurospora crassa</u> NADP-GDHs

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            5'-GTT GAG CCT GAG CGC GTG ATC ACC TTC CGC GTG TCC TGG CTG GAC GAC GCC GGC AAC CTG CAG GTC   66
Chlorella      V   E   P   E   R   V   I   T   F   R   V   S   W   L   D   D   A   G   N   L   Q   V
Escherichia    V   E   P   E   R   V   I   Q   F   R   V   V   W   V   D   D   R   N   Q   I   Q   V
Neurospora     S   I   P   E   R   V   I   Q   F   R   V   V   W   E   D   D   N   G   N   Y   Q   V

               AAC CGC GGC TTC CGC GTG CAG TAC TCG TCC GCC ATC GGC CCC TAC AAG GGC GGC CTG CGC TTC CAC  132
               N   H   G   F   R   V   Q   Y   S   S   A   I   G   P   Y   K   G   G   L   R   F   H
               N   R   A   W   R   V   Q   F   S   S   A   I   G   P   Y   K   G   G   M   R   F   H
               N   R   G   Y   R   V   Q   F   N   S   A   L   G   P   Y   K   G   G   L   R   L   H

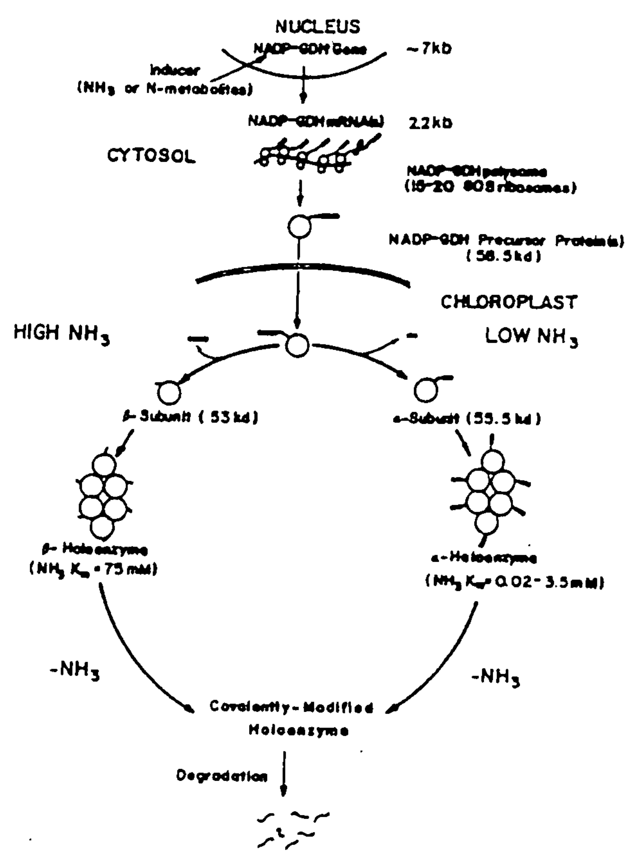
               CCC TCC GTG AAC CTG TCC ATC ATG AAG TTC CTT GCC TTT GAG CAG ATC TTC AAG AAC AGC CTG ACC  198
               P   S   V   N   L   S   I   M   K   F   L   A   F   E   Q   I   F   K   N   S   L   T
               P   S   V   N   L   S   I   L   K   F   L   G   F   E   Q   T   F   K   N   A   L   T
               P   S   V   N   L   S   I   L   K   F   L   G   F   E   Q   I   F   K   N   A   L   T

               ACC CTG CCC ATG GGC GGC GGC AAG GGC GGC TCC GAC TTC GAC CCC AAG GGC AAG AGC GAC GCG GAG  264
               T   L   P   M   G   G   G   K   G   G   S   D   F   D   P   K   G   K   S   D   A   E
               T   L   P   M   G   G   G   K   G   G   S   D   F   D   P   K   G   K   S   E   G   E
               G   L   S   M   G   G   G   K   G   G   A   D   F   D   P   K   G   K   S   D   A   E

               GTG ATG CGC TTC TGC CAG TCC TTC ATG ACC GAG CTG CAG CGC CAC ATC AGC TAC GTG CAG GAC GTG  330
               V   M   R   F   C   Q   S   F   M   T   E   L   Q   R   H   I   S   Y   V   Q   D   V
               V   M   R   F   C   Q   A   L   M   T   E   L   Y   R   H   L   G   A   D   T   D   V
               I   R   R   F   C   C   A   F   M   A   E   L   H   K   H   I   G   A   D   T   D   V

               CCC GCC GGC GAC ATC GGC GTG GGC GCG CGC-3'                                              360
               P   A   G   D   I   G   V   G   A   R
               P   A   G   D   I   G   V   G   G   R       E. coli   77% homologous
               P   A   G   D   I   G   V   G   F   R       N. crassa 73% homologous
```

MODEL FOR NADP-GDH REGULATION



5

BIBLIOGRAPHY

1. Miflin, B.J., and P.J. Lea (1976) Photochemistry 15:873.
2. Miflin, B.J., and P.J. Lea (1980) In, The Biochemistry of Plants, B.J. Miflin (ed.), Academic Press, p. 169.
3. ~~Keys, A.J., Bird, I.F., Cornelius, M.F., Lea, P.J., Wallsgrove, R.M., and B.J. Miflin (1978) Nature 275:741.~~
4. ~~Ogren, W.L. (1984) Annu. Rev. Plant Physiol. 35:415.~~
5. Givan, C.V., Joy, K.W., and L.A. Kleczkowski (1988) TIBS 13:433.
6. Wootton, J.C., and M.J. McPherson (1984) In, The Genetic Manipulation of Plants and Its Application to Agriculture, P.J. Lea and G.R. Stewart (eds.), Cavendon Press, p. 89.
7. Stewart, G.R., Mann, A.F., and P.A. Fentem (1980) In, The Biochemistry of Plants, Vol. 5, Academic Press, p. 271.
8. Meredith, M.J., Gronostajski, R.M., and R.R. Schmidt (1978) Plant Physiol. 61:967.
9. Schmidt, R.R., Turner, K.J., Bascomb, N.F., Thurston, C.F., Lynch, J.J., Molin, W.T., and A.T. Yeung (1982) In, Genetic Expression in The Cell Cycle, K.S. McCarty, Sr., and G.M. Padilla (eds.), Academic Press, p. 199.
10. Yeung, A.T., Turner, K.J., Bascomb, N.F., and R.R. Schmidt (1981) Anal. Biochem. 110:216.
11. Bascomb, N.F., and R.R. Schmidt (1987) Plant Physiol. 83:75.
12. Cock, J.M., Roof, L.L., Bascomb, N.F., Gehrke, C.W., Kuo, K.C., and R.R. Schmidt (1990) J. Phycol. 26, 361.
13. Schmidt, R.R. (1990) In, New Directions in Biological Control, UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 112, R. Baker and P. Dunn (eds.), Alan R. Liss, p. 1-22.
14. Cock, J.M., Kim, K.-D., and R.R. Schmidt (1990) Sequence of a nuclear gene with many introns encoding two chloroplastic NADP-specific glutamate dehydrogenase isoenzymes in Chlorella sorokiniana. Plant Mol. Biol., submitted December 1990.
15. Cock, J.M., Kim, K.-D., Miller, P.W., and R.R. Schmidt (1990) Sequence and induction pattern of a mRNA possibly encoding two ammonium inducible NADP-specific glutamate dehydrogenase isoenzymes in Chlorella sorokiniana. Plant Mol. Biol., submitted December 1990.
16. Nadal-Ginard, B., Gallego, M.E., and A. Andreadis (1987) In, Genetic Engineering, Vol. 9, J.E. Setlow (ed), Plenum Press, p. 249.
17. Werneke, J.M., Chatfield, J.M., and W.L. Ogren (1989) The Plant Cell 1:815.
18. Perlman, D., Raney, R., and H.O. Halvorson (1984) Mol. Cell Biol. 4:1682.
19. Innis, M.A., Gelfand, D.H., Sninsky, J.J., and T.J. White (1990) PCR Protocols: A Guide to Methods and Applications, Academic Press, pp. 482.
20. Bascomb, N.F., Prunkard, D.E., and R.R. Schmidt (1987) Plant Physiol. 83:85.
21. Bascomb, N.F., and R.R. Schmidt (1990) ATP and calcium requirement for inactivation and degradation of chloroplastic NADP-specific glutamate dehydrogenase in permeabilized cells and cell-free extracts of Chlorella sorokiniana. Plant Physiol., in preparation.
22. Somerville, C.R., and W.L. Ogren (1980) Nature 286:257.
23. Wallsgrove, R.M., Turner, J.C., Hall, N.P., Kendall, A.C., and S.W.J. Bright (1987) Plant Physiol. 83:155.
24. McNally, S.F., Hirel, B., Gadal, P., Mann, A.F., and G.R. Stewart (1983) Plant

Physiol. 72:22.
25. Edwards, J.W., and Coruzzi, G.M. (1989) The Plant Cell 1:241.
26. Walker, E.L., and G.M. Coruzzi (1989) Plant Physiol. 91:702.
27. Lightfoot, D.A., Green, N.K., and J.V. Cullimore (1988) Plant Mol. Biol. 11:191.
28. ~~Forde, B.G., Day, H.M., Turton, J.F., Wen-jun, S., Cullimore, J.V., and J.E. Oliver (1989) The Plant Cell 1:391.~~
29. ~~Estelle, M.A., and C.R. Somerville (1986) Trends in Genetics 2:89.~~
30. Bevan, M., and A. Goldsbrough (1988) In, Genetic Engineering, Vol. 10, J.K. Setlow (ed.), Plenum Press, p. 123.
31. Lloyd, A.M., Barnason, A.R., Rogers, S.G., Byrne, M.C., Fraley, R.T., and R.B. Horsch (1986) Science 234:464.
32. Feldman, K.A., and M.D. Marks (1987) Mol. Gen. Genet. 208:1.
33. Feldmann, K.A., Marks, M.D., Christianson, M.L., and R.S. Quatrano (1989) Science 243:1351.
34. Valvekens, D., vanMontagu, M., and M. vanLijsebettens (1988) Proc. Natl. Acad. Sci. USA 85:5536.
35. Bascomb, N.F., Turner, K.J., and R.R. Schmidt (1986) Plant Physiol. 81:527.
36. Prunkard, D.E., Bascomb, N.F., Robinson, R.W., and R.R. Schmidt (1986) Plant Physiol. 81:349.
37. Prunkard, D.E., Bascomb, N.F., Molin, W.T., and R.R. Schmidt (1986) Plant Physiol. 81:413.
38. Keegstra, K., and L.J. Olsen (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:471.

BIOGRAPHICAL SKETCH

Robert R. Schmidt, Ph.D. — Birthdate: February 18, 1933
Principal Investigator

Current Position: Graduate Research Professor

Education:

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	FIELD OF STUDY
Virginia Polytechnic Institute & State University	B.S.	1955	Plant and Microbiol. Sci.
University of Maryland	M.S.	1957	Plant Physiology
Virginia Polytechnic Institute & State University	Ph.D.	1961	Biochemistry

M.S. Degree Advisor: Dr. R.W. Krauss
Ph.D. Degree Advisor: Dr. K.W. King (Deceased)

Employment/Experience:
1961-64 Assistant Prof., Dept. Biochemistry & Nutrition, VPI&SU, Blacksburg, VA.
1964-67 Associate Prof., Dept. Biochemistry & Nutrition, VPI&SU, Blacksburg, VA
1967-80 Professor, Dept. Biochemistry & Nutrition, VPI&SU, Blacksburg, VA

Sept. 1976 - June 1977, Visiting Professor of Biological Sciences, in laboratory of Dr. R.T. Schimke, Department of Biological Science, Stanford University, Calif. I learned immunological and nucleic acid techniques used in measurements of specific enzyme synthesis and degradation; mRNA isolation and purification; cDNA synthesis and hybridization.
Dec. 1980 - June 1982, Professor and Chairman, Dept. Microbiology & Cell Science, University of Florida, Gainesville, FL.
July 1982 - present, GRADUATE RESEARCH PROFESSOR (i.e., highest rank that a Full Professor can hold at the Univ. of Florida) in same department.

Honors/Awards:
Certificate of Teaching Excellence (1978); American Society of Biological Chemists (1967) Sigma Xi Graduate Research Award (1961); Phi Kappa Phi (1955); Phi Sigma Undergraduate Research Award (1954); Bausch and Lomb Honorary Science Award (1951).

Graduate Research/Teaching Experience:
In the last 29 years, I have supervised graduate students in my laboratory to 35 advanced degrees; Supervised 13 postdoctoral research associates; 4 visiting professors, and 5 laboratory technicians. I have supported these personnel primarily on grants from NIH, NSF, USDA, and NASA.
I currently have a research group which consists of 4 graduate students, a senior laboratory technician, and a Visiting Full Professor on a 12 month sabbatical leave.

For 13 years, I taught a 2-quarter advanced graduate-level course, entitled Genetic and Metabolic Control. In this course, regulation of gene expression in both procaryotes and eucaryotes was discussed in great detail with emphasis on current techniques and experimental approaches in molecular biology and nucleic acid biochemistry.

I currently teach a major section of the departmental graduate core course in the area of biochemistry and molecular biology of nitrogen assimilation.

~~Publications:~~

I currently have 51 regular research publications; 8 chapters in books, two technical comments, and several papers in preparation. Ten selected publications related to this project are listed below:

1. Cock, J.M., Kim, K.D., Miller, P.W., and Schmidt, R.R. (1990) Nucleotide sequence and ammonium induction pattern of the mRNA possibly encoding two chloroplastic NADP-specific glutamate dehydrogenase isoenzymes in Chlorella sorokiniana. Plant Molec. Biol., submitted.

2. Cock, J.M., Kim, K.D., Miller, P.W., and Schmidt, R.R. (1990) Sequence of a nuclear gene with many introns encoding two chloroplastic ammonium-inducible glutamate dehydrogenase isoenzymes in Chlorella sorokiniana. Plant Molec. Biol., submitted.

3. Cock, J.M., Roof, L.L., Bascomb, N.F., Gehrke, C.W., Kuo, K.C., and Schmidt, R.R. (1990) Restriction enzyme analysis and cloning of high molecular weight genomic DNA isolated from Chlorella sorokiniana (Chlorophyta). J. Phycol. 26:361-367.

4. Schmidt, R.R. (1990) In, New Directions in Biological Control (UCLA Symposia on Molecular and Cellular Biology, New Series, Vol. 112; Baker, R., and Dunn, P., eds.), Alan R. Liss, New York. Investigation of mechanisms: the key to successful use of biotechnology, pp 1-22.

5. Cock, J.M., and Schmidt, R.R. (1989) A glutamate dehydrogenase gene sequence. Nucleic Acids Research 17:10500.

6. Bascomb, N.F., Prunkard, D.E., and Schmidt, R.R. (1987) Different rates of synthesis and degradation of two chloroplastic ammonium-inducible NADP-specific glutamate dehydrogenase isoenzymes during induction and deinduction in Chlorella sorokiniana cells. Plant Physiol. 83, 85-91.

7. Bascomb, N.F., and Schmidt, R.R. (1987) Purification and partial kinetic and physical characterization of two chloroplast-localized NADP-specific glutamate dehydrogenase isoenzymes and their preferential accumulation in Chlorella sorokiniana cells cultured at low or high ammonium levels. Plant Physiol. 83, 75-84.

8. Bascomb, N.F., Turner, K.J., and Schmidt, R.R. (1986) Specific polysome immunoadsorption to purify an ammonium-inducible glutamate dehydrogenase mRNA from Chlorella sorokiniana and synthesis of full length double-stranded cDNA from the purified mRNA. Plant Physiol. 81, 527-532.

9. ~~Prunkard, D.E., Bascomb, N.F., Molin, W.T., and Schmidt, R.R. (1986) Effect of~~

different carbon sources on the ammonium induction of different forms of NADP-specific glutamate dehydrogenase in Chlorella sorokiniana cells cultured in the light and dark. Plant Physiol. 81, 413-422.

10. Prunkard, D.E., Bascomb, N.F., Robinson, R.W. and Schmidt, R.R. (1986) Evidence for chloroplastic localization of an ammonium-inducible glutamate dehydrogenase ~~and synthesis of its subunit from a cytosolic precursor-protein in Chlorella sorokiniana. Plant Physiol. 81, 349-355.~~

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FIRST YEAR

APPENDIX V

(SEE INSTRUCTIONS ON REVERSE BEFORE COMPLETING

SUMMARY PROPOSAL BUDGET

ORGANIZATION University of Florida/Division of Sponsored Research		FOR NSF USE ONLY PROPOSAL NO.	DURATION (MONTHS) Proposed	Granted
PRINCIPAL INVESTIGATOR/PROJECT DIRECTOR Robert R. Schmidt		AWARD NO.		

A. SENIOR PERSONNEL: PI/PD, Co-PI's, Faculty and Other Senior Associates (List each separately with title, A.6. show number in brackets)	NSF Funded Person-mos. CAL.	ACAD	SUMR	Funds Requested By Proposer	Funds Granted By NSF (If Different)
~~1. R.R. Schmidt, Graduate Research Professor~~				~~$ None~~	$
2.					
3.					
4.					
5. ( ) OTHERS (LIST INDIVIDUALLY ON BUDGET EXPLANATION PAGE)					
6. ( ) TOTAL SENIOR PERSONNEL (1-5)					
B. OTHER PERSONNEL (SHOW NUMBERS IN BRACKETS)					
1. ( ) POST DOCTORAL ASSOCIATES					
2. ( ) OTHER PROFESSIONALS (TECHNICIAN, PROGRAMMER, ETC.)					
3. (3) GRADUATE STUDENTS (Ph.D. candidates)				32,805	
4. (2) UNDERGRADUATE STUDENTS Part-time @ 4.75/hr.				2,000	
5. ( ) SECRETARIAL CLERICAL					
6. ( ) OTHER					
TOTAL SALARIES AND WAGES (A+B)				34,805	
C. FRINGE BENEFITS (IF CHARGED AS DIRECT COSTS)					
TOTAL SALARAIES, WAGES AND FRINGE BENEFITS (A+B+C)				34,805	
D. PERMANENT EQUIPMENT (LIST ITEM AND DOLLAR AMOUNT FOR EACH ITEM EXCEEDING $1,000:) Small equipment items costing less than $500 ea., e.g., pumps, mixers, Plexiglas electrophoresis chambers, Eppendorf automatic pipettes, etc.					
TOTAL PERMANENT EQUIPMENT				2,000	
E. TRAVEL 1. DOMESTIC (INCL. CANADA AND U.S. POSSESSIONS) National meetings				2,000	
2. FOREIGN					
F. PARTICIPANT SUPPORT COSTS 1. STIPENDS $ ______ 2. TRAVEL ______ 3. SUBSISTENCE ______ 4. OTHER ______					
( ) TOTAL PARTICIPANT COSTS					
G. OTHER DIRECT COSTS					
1. MATERIALS AND SUPPLIES				17,000	
2. PUBLICATION COSTS/DOCUMENTATION/DISSEMINATION				1,500	
3. CONSULTANT SERVICES					
4. COMPUTER (ADPE) SERVICES					
5. SUBCONTRACTS					
6. OTHER (Repair of major equipment items)				2,500	
TOTAL OTHER DIRECT COSTS				21,000	
H. TOTAL DIRECT COSTS (A THROUGH G)				59,805	
I. INDIRECT COSTS (SPECIFY RATE AND BASE) 45% modified total direct costs					
TOTAL INDIRECT COSTS				26,912	
J. TOTAL DIRECT AND INDIRECT COSTS (H + I)				86,717	
K. RESIDUAL FUNDS (IF FOR FURTHER SUPPORT OF CURRENT PROJECTS SEE GPM 252 AND 253)					
L. AMOUNT OF THIS REQUEST (J) OR (J MINUS K)				$ 86,717	$

PI/PD TYPED NAME & SIGNATURE* Robert R. Schmidt	DATE	FOR NSF USE ONLY INDIRECT COST RATE VERIFICATION		
INST. REP. TYPED NAME & SIGNATURE*	DATE	Date Checked	Date of Rate Sheet	Initials-DGC

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*SIGNATURES REQUIRED ONLY FOR REVISED BUDGET (GPM 233)

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SECOND YEAR

APPENDIX V

(SEE INSTRUCTIONS ON REVERSE BEFORE COMPLETING

SUMMARY PROPOSAL BUDGET

FOR NSF USE ONLY		
PROPOSAL NO.	DURATION (MONTHS) Proposed	Granted
AWARD NO.		

ORGANIZATION
University of Florida/Division of Sponsored Research

PRINCIPAL INVESTIGATOR/PROJECT DIRECTOR
Robert R. Schmidt

A. SENIOR PERSONNEL: PI/PD, Co-PI's, Faculty and Other Senior Associates (List each separately with title, A.6. show number in brackets)	NSF Funded Person-mos. CAL.	ACAD	SUMR	Funds Requested By Proposer	Funds Granted By NSF (If Different)
1. ~~R.R. Schmidt, Graduate Research Professor (PI)~~				~~$ None~~	$
~~2.~~					
~~3.~~					
4.					
5. ( ) OTHERS (LIST INDIVIDUALLY ON BUDGET EXPLANATION PAGE)					
6. ( ) TOTAL SENIOR PERSONNEL (1-5)					
B. OTHER PERSONNEL (SHOW NUMBERS IN BRACKETS)					
1. ( ) POST DOCTORAL ASSOCIATES					
2. ( ) OTHER PROFESSIONALS (TECHNICIAN, PROGRAMMER, ETC.)					
3. (3) GRADUATE STUDENTS (Ph.D. candidates)				34,445	
4. (2) UNDERGRADUATE STUDENTS Part-time @ 4.75/hr				2,000	
5. ( ) SECRETARIAL CLERICAL					
6. ( ) OTHER					
TOTAL SALARIES AND WAGES (A+B)				36,445	
C. FRINGE BENEFITS (IF CHARGED AS DIRECT COSTS)					
TOTAL SALARAIES, WAGES AND FRINGE BENEFITS (A+B+C)				36,445	
D. PERMANENT EQUIPMENT (LIST ITEM AND DOLLAR AMOUNT FOR EACH ITEM EXCEEDING $1,000:) Small equipment items costing less than $500 ea.					
TOTAL PERMANENT EQUIPMENT				2,000	
E. TRAVEL 1. DOMESTIC (INCL. CANADA AND U.S. POSSESSIONS) National meetings				2,000	
2. FOREIGN					
F. PARTICIPANT SUPPORT COSTS 1. STIPENDS $ 2. TRAVEL 3. SUBSISTENCE 4. OTHER					
( ) TOTAL PARTICIPANT COSTS					
G. OTHER DIRECT COSTS					
1. MATERIALS AND SUPPLIES				17,850	
2. PUBLICATION COSTS/DOCUMENTATION/DISSEMINATION				1,500	
3. CONSULTANT SERVICES					
4. COMPUTER (ADPE) SERVICES					
5. SUBCONTRACTS					
6. OTHER (Repair of major equipment items)				2,500	
TOTAL OTHER DIRECT COSTS				21,850	
H. TOTAL DIRECT COSTS (A THROUGH G)				62,295	
I. INDIRECT COSTS (SPECIFY RATE AND BASE) 45% modified total direct costs					
TOTAL INDIRECT COSTS				28,033	
J. TOTAL DIRECT AND INDIRECT COSTS (H + I)				90,328	
K. RESIDUAL FUNDS (IF FOR FURTHER SUPPORT OF CURRENT PROJECTS SEE GPM 252 AND 253)					
L. AMOUNT OF THIS REQUEST (J) OR (J MINUS K)				$90,328	$

PI/PD TYPED NAME & SIGNATURE*	DATE	FOR NSF USE ONLY		
Robert R. Schmidt		INDIRECT COST RATE VERIFICATION		
INST. REP. TYPED NAME & SIGNATURE*	DATE	Date Checked	Date of Rate Sheet	Initials-DGC

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APPENDIX V

(SEE INSTRUCTIONS ON REVERSE BEFORE COMPLETING

SUMMARY PROPOSAL BUDGET

THIRD YEAR

	FOR NSF USE ONLY	
ORGANIZATION University of Florida/Division of Sponsored Research	PROPOSAL NO.	DURATION (MONTHS) Proposed / Granted
PRINCIPAL INVESTIGATOR/PROJECT DIRECTOR Robert R. Schmidt	AWARD NO.	

A. SENIOR PERSONNEL: PI/PD, Co-PI's, Faculty and Other Senior Associates (List each separately with title, A.6. show number in brackets)	NSF Funded Person-mos. CAL.	ACAD	SUMR	Funds Requested By Proposer	Funds Granted By NSF (If Different)
1. ~~R.R. Schmidt, Graduate Research Professor (PI)~~				$ None	$
~~2.~~					
~~3.~~					
4.					
5. ( ) OTHERS (LIST INDIVIDUALLY ON BUDGET EXPLANATION PAGE)					
6. ( ) TOTAL SENIOR PERSONNEL (1-5)					
B. OTHER PERSONNEL (SHOW NUMBERS IN BRACKETS)					
1. ( ) POST DOCTORAL ASSOCIATES					
2. ( ) OTHER PROFESSIONALS (TECHNICIAN, PROGRAMMER, ETC.)					
3. (3) GRADUATE STUDENTS (Ph.D. candidates)				36,167	
4. (2) UNDERGRADUATE STUDENTS Part-time @ $5.00/hr				2,500	
5. ( ) SECRETARIAL CLERICAL					
6. ( ) OTHER					
TOTAL SALARIES AND WAGES (A+B)				38,667	
C. FRINGE BENEFITS (IF CHARGED AS DIRECT COSTS)					
TOTAL SALARAIES, WAGES AND FRINGE BENEFITS (A+B+C)				38,667	
D. PERMANENT EQUIPMENT (LIST ITEM AND DOLLAR AMOUNT FOR EACH ITEM EXCEEDING $1,000:) Small equipment items costing less than $500 ea.					
TOTAL PERMANENT EQUIPMENT				2,000	
E. TRAVEL 1. DOMESTIC (INCL. CANADA AND U.S. POSSESSIONS) National meetings				2,000	
2. FOREIGN					
F. PARTICIPANT SUPPORT COSTS 1. STIPENDS $ ____ 2. TRAVEL ____ 3. SUBSISTENCE ____ 4. OTHER ____					
( ) TOTAL PARTICIPANT COSTS					
G. OTHER DIRECT COSTS					
1. MATERIALS AND SUPPLIES				18,743	
2. PUBLICATION COSTS/DOCUMENTATION/DISSEMINATION				1,500	
3. CONSULTANT SERVICES					
4. COMPUTER (ADPE) SERVICES					
5. SUBCONTRACTS					
6. OTHER (Repair of major equipment items)				2,500	
TOTAL OTHER DIRECT COSTS				22,743	
H. TOTAL DIRECT COSTS (A THROUGH G)				65,410	
I. INDIRECT COSTS (SPECIFY RATE AND BASE) 45% modified total direct costs					
TOTAL INDIRECT COSTS				29,435	
J. TOTAL DIRECT AND INDIRECT COSTS (H + I)				94,845	
K. RESIDUAL FUNDS (IF FOR FURTHER SUPPORT OF CURRENT PROJECTS SEE GPM 252 AND 253)					
L. AMOUNT OF THIS REQUEST (J) OR (J MINUS K)				$ 94,845	$

PI/PD TYPED NAME & SIGNATURE*	DATE	FOR NSF USE ONLY		
Robert R. Schmidt		INDIRECT COST RATE VERIFICATION		
INST. REP. TYPED NAME & SIGNATURE*	DATE	Date Checked	Date of Rate Sheet	Initials-DGC

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*SIGNATURES REQUIRED ONLY FOR REVISED BUDGET (GPM 233)

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FOURTH YEAR

APPENDIX V

(SEE INSTRUCTIONS ON REVERSE BEFORE COMPLETING

SUMMARY PROPOSAL BUDGET

FOR NSF USE ONLY		
PROPOSAL NO.	DURATION (MONTHS) Proposed	Granted
AWARD NO.		

ORGANIZATION
University of Florida/Division of Sponsored Research

PRINCIPAL INVESTIGATOR/PROJECT DIRECTOR
Robert R. Schmidt

A. SENIOR PERSONNEL: PI/PD. Co-PI's, Faculty and Other Senior Associates (List each separately with title, A.6. show number in brackets)	NSF Funded Person-mos. CAL.	ACAD	SUMR	Funds Requested By Proposer	Funds Granted By NSF (If Different)
1. R.R. Schmidt, Graduate Research Professor (PI)				$ None	$
2.					
3.					
4.					
5. ( ) OTHERS (LIST INDIVIDUALLY ON BUDGET EXPLANATION PAGE)					
6. ( ) TOTAL SENIOR PERSONNEL (1-5)					
B. OTHER PERSONNEL (SHOW NUMBERS IN BRACKETS)					
1. ( ) POST DOCTORAL ASSOCIATES					
2. ( ) OTHER PROFESSIONALS (TECHNICIAN; PROGRAMMER, ETC.)					
3. (3) GRADUATE STUDENTS (Ph.D. candidates)				37,975	
4. (2) UNDERGRADUATE STUDENTS Part-time @ $5.00/hr				2,500	
5. ( ) SECRETARIAL CLERICAL					
6. ( ) OTHER					
TOTAL SALARIES AND WAGES (A+B)				40,475	
C. FRINGE BENEFITS (IF CHARGED AS DIRECT COSTS)					
TOTAL SALARAIES, WAGES AND FRINGE BENEFITS (A+B+C)				40,475	
D. PERMANENT EQUIPMENT (LIST ITEM AND DOLLAR AMOUNT FOR EACH ITEM EXCEEDING $1,000:) Small equipment items costing less than $500 ea.					
TOTAL PERMANENT EQUIPMENT				2,000	
E. TRAVEL 1. DOMESTIC (INCL. CANADA AND U.S. POSSESSIONS) National meetings				2,000	
2. FOREIGN					
F. PARTICIPANT SUPPORT COSTS 1. STIPENDS $ ____ 2. TRAVEL ____ 3. SUBSISTENCE ____ 4. OTHER ____					
( ) TOTAL PARTICIPANT COSTS					
G. OTHER DIRECT COSTS					
1. MATERIALS AND SUPPLIES				19,680	
2. PUBLICATION COSTS/DOCUMENTATION/DISSEMINATION				1,500	
3. CONSULTANT SERVICES					
4. COMPUTER (ADPE) SERVICES					
5. SUBCONTRACTS					
6. OTHER (Repair of major equipment items)				2,500	
TOTAL OTHER DIRECT COSTS				23,680	
H. TOTAL DIRECT COSTS (A THROUGH G)				68,155	
I. INDIRECT COSTS (SPECIFY RATE AND BASE) 45% modified total direct costs					
TOTAL INDIRECT COSTS				30,670	
J. TOTAL DIRECT AND INDIRECT COSTS (H + I)				98,825	
K. RESIDUAL FUNDS (IF FOR FURTHER SUPPORT OF CURRENT PROJECTS SEE GPM 252 AND 253)					
L. AMOUNT OF THIS REQUEST (J) OR (J MINUS K)				$ 98,825	$

PI/PD TYPED NAME & SIGNATURE*	DATE	FOR NSF USE ONLY		
Robert R. Schmidt		INDIRECT COST RATE VERIFICATION		
INST. REP. TYPED NAME & SIGNATURE*	DATE	Date Checked	Date of Rate Sheet	Initials-DGC

NSF Form 1030 (8/90) *Supersedes All Previous Editions*

*SIGNATURES REQUIRED ONLY FOR REVISED BUDGET (GPM 233)

Best Available Copy

APPENDIX V

(SEE INSTRUCTIONS ON REVERSE BEFORE COMPLETING

SUMMARY PROPOSAL BUDGET

CUMULATIVE BUDGET

ORGANIZATION University of Florida/Division of Sponsored Research				PROPOSAL NO.	DURATION (MONTHS) Proposed	Granted
PRINCIPAL INVESTIGATOR/PROJECT DIRECTOR Robert R. Schmidt				AWARD NO.		
A. SENIOR PERSONNEL: PI/PD, Co-PI's, Faculty and Other Senior Associates (List each separately with title, A.6. show number in brackets)	NSF Funded Person-mos. CAL	ACAD	SUMR	Funds Requested By Proposer	Funds Granted By NSF (If Different)	
1. ~~R. R. Schmidt, Graduate Research Professor (PI)~~				~~$ None~~	$	
2.						
3.						
4.						
5. ( ) OTHERS (LIST INDIVIDUALLY ON BUDGET EXPLANATION PAGE)						
6. ( ) TOTAL SENIOR PERSONNEL (1-5)						
B. OTHER PERSONNEL (SHOW NUMBERS IN BRACKETS)						
1. ( ) POST DOCTORAL ASSOCIATES						
2. ( ) OTHER PROFESSIONALS (TECHNICIAN, PROGRAMMER, ETC.)						
3. (3) GRADUATE STUDENTS				141,392		
4. (2) UNDERGRADUATE STUDENTS				9,000		
5. ( ) SECRETARIAL CLERICAL						
6. ( ) OTHER						
TOTAL SALARIES AND WAGES (A+B)				150,392		
C. FRINGE BENEFITS (IF CHARGED AS DIRECT COSTS)						
TOTAL SALARAIES, WAGES AND FRINGE BENEFITS (A+B+C)				150,392		
D. PERMANENT EQUIPMENT (LIST ITEM AND DOLLAR AMOUNT FOR EACH ITEM EXCEEDING $1,000:) Small equipment items costing less than $500 ea., e.g., pumps, mixers, Plexiglas electrophoresis chambers, Eppendorf automatic pipettes, etc.						
TOTAL PERMANENT EQUIPMENT				8,000		
E. TRAVEL 1. DOMESTIC (INCL. CANADA AND U.S. POSSESSIONS)				8,000		
2. FOREIGN						
F. PARTICIPANT SUPPORT COSTS 1. STIPENDS $ 2. TRAVEL 3. SUBSISTENCE 4. OTHER						
( ) TOTAL PARTICIPANT COSTS						
G. OTHER DIRECT COSTS						
1. MATERIALS AND SUPPLIES				73,273		
2. PUBLICATION COSTS/DOCUMENTATION/DISSEMINATION				6,000		
3. CONSULTANT SERVICES						
4. COMPUTER (ADPE) SERVICES						
5. SUBCONTRACTS						
6. OTHER (Repair of major equipment items)				10,000		
TOTAL OTHER DIRECT COSTS				89,273		
H. TOTAL DIRECT COSTS (A THROUGH G)				255,665		
I. INDIRECT COSTS (SPECIFY RATE AND BASE) 45% modified total direct costs						
TOTAL INDIRECT COSTS				115,050		
J. TOTAL DIRECT AND INDIRECT COSTS (H + I)				370,715		
K. RESIDUAL FUNDS (IF FOR FURTHER SUPPORT OF CURRENT PROJECTS SEE GPM 252 AND 253)						
L. AMOUNT OF THIS REQUEST (J) OR (J MINUS K)				$ 370,715	$	

PI/PD TYPED NAME & SIGNATURE* Robert R. Schmidt	DATE	FOR NSF USE ONLY INDIRECT COST RATE VERIFICATION		
INST. REP. TYPED NAME & SIGNATURE*	DATE	Date Checked	Date of Rate Sheet	Initials-DGC

NSF Form 1030 (8/90) *Supersedes All Previous Editions*

*SIGNATURES REQUIRED ONLY FOR REVISED BUDGET (GPM 233)

BUDGET JUSTIFICATION

Personnel:

1. Principal Investigator:
   ~~Dr. Schmidt will spend 25% of his time on this project. No salary funds are requested.~~

2. Three Graduate Research Assistants:
   Mr. Richard Hutson received a B.S. degree in Microbiology from the Virginia Polytechnic Institute and State University, and will receive the M.S. degree in molecular biology under my direction in He will pursue his Ph.D. in my laboratory.

   Mr. Philip Miller received a M.S. degree in Genetics from Appalachian State University and joined my laboratory Spring Semester 1990 and is pursuing his Ph.D. in molecular biology under my direction.

   Ms. Brenda Russell received a M.S. degree in Microbiology from the Virginia Polytechnic Institute & State University and joined my laboratory Summer Semester 1990 and is pursuing her Ph.D. in molecular biology under my direction.

   Each of these graduate students is currently working on aspects of the molecular biology of the Chlorella and Arabidopsis project. Their continued work in this area requires an extramural grant.

3. Laboratory Aids:
   Part-time undergraduate-student employees are required to wash and/or sterilize the large volume of dirty laboratory glassware and culture tubes, etc. generated by an active research group. These part-time laboratory aids also are involved in the general laboratory maintenance required in a biochemistry/molecular biology laboratory.

4. Laboratory Technician (State funded):
   Ms. Waltraud Dunn, a senior level state-funded laboratory technician will devote approximately 25% of her time to this project with no funds requested for her salary from the NSF.

The salaries of the graduate students will be increased by 5% each year. There is a Graduate Student Union at the University of Florida that negotiates raises each year which range between 4% and 6%.

Permanent Equipment:

At the present time, we have (or have access to) all of the major equipment items required for this project. However, we routinely need to purchase small permanent equipments items which cost less than $500, i.e., automatic pipettes, electrophoresis chambers, dialysis chambers, pumps, thermoregulators, magnetic stirrers, heaters, etc.

Travel:

Funds are requested to give talks/posters at the national meetings of the American Society of Biological Chemists, American Society of Plant Physiologists, and the American Society of Microbiology. The principal investigator, and graduate students will be attendees ~~provided talks/posters are presented.~~

Materials and Supplies (per year):

1. Radioactive compounds, enzyme substrates, protein standards, restriction enzymes and other recombinant DNA reagents and linkers, translation assay components, Protein A, and other biochemical reagents, etc. $11,000

2. Chromatography, electrophoresis, chromatofocusing columns, gels, packings, affinity resins, cellulose nitrate paper and other derivatized papers, polybuffers, etc. $3,000

3. Glassware, plasticware, scintillation vials, Eppendorf pipettes tips, distilled H_2O dionizer cartridges, culture tubes, microcentrifuge tubes, liquid nitrogen, carbon dioxide, argon, X-ray film, etc. $3,000

   Because of the rapid increase in costs of biochemical and molecular biology reagents, a 5% increase per year is budgeted.

Publication Costs/Page Charges:

The funds are requested for page costs and also for making photographs of gels, autoradiograms, etc. and for preparation of figures for publication. With research progressing so rapidly, it is anticipated that equal funding will be required each year for publication related costs.

Other Direct Costs:

The costs for repairs of power supplies, centrifuges, low temperature freezers, Coulter cell counter, spectrophotometer, freezer drier, fraction collectors, etc. routinely costs a minimum of $2,500 per year.

Current and Pending Support for Research and Education in Science and Engineering

The following information should be provided for each investigator and other senior personnel. Failure to provide this information may delay consideration of this proposal.

1. Name of Principal Investigator	Source of Support	Project Title	Award Amount (or Annual rate)	Period Covered by Award	* Person-months or % of Effort Committed to the Project: ACAD.	SUMM	CAL.	Location of Research
A. *Current Support* List—if none, report none	USDA Competitive Grants Program #89-37262-4843	Nuclear Gene Encoding Two NH_4^+ Inducible Chloroplastic Isoenzymes	$90,000				25%	Dept. of Microbiology & Cell Science, Univ. Florida
B. *Proposals Pending* 1. List this proposal	NSF	"Develop. Transgenic Plants Requiring Less ATP for NH_4^+ Assimilation"	$370,715	48 mo.			25%	Same as above
2. *Other pending proposals,* including renewal applications. If none, report none.	NIH	"Gene-Enzyme Con- in Eukaryotic Cell Cycle"	$453,605	48 mo.			30%	Same as above.
3. *Proposals planned to be submitted in near future.* If none, report none.	NSF	Regulation of Glutamate Synthetase Genes in a C_4 Grass	approx. $300,000	36 mo.			20%	Same as above.
II. Name of co-principal investigator and/or faculty associate. A. ______ B. ______								
III. Transfer of Support If this project has previously been funded by another agency, please list and furnish information for immediately preceding funding period.								
IV. Other agencies to which this proposal has been/will be submitted								

USE ADDITIONAL SHEETS AS NECESSARY

*No salary funds requested for PI for % effort.

NSF Form 1239 (8/90)

APPENDIX

Facilities

Dr. Schmidt has a laboratory of 1,600 sq. ft. which has essentially all of the equipment required for modern research in plant and microbial biochemistry and molecular biology. Typical equipment items include: two large nucleotide sequencing apparatus, 1 - Pharmacia Fast Protein Chromatograph with different types of analytical columns, multiple units for analytical and preparative slab-gel electrophoresis, transilluminator with Polaroid camera, fraction collectors and monitors, density-gradient former and fractionators, 1 - ultracentrifuge, several refrigerated centrifuges, -70°C freezer, Gilford recording spectrophotometer, a laboratory personal computer connected to university VAX, etc. In addition, the department has scintillation counters, an oligonucleotide synthesizer, electron microscopes, etc. The university Interdisciplinary Center for Biotechnology Research (ICBR) has the protein sequencer, amino acid analyzers, LKB laser densitometer, DNA sequencer, DNA synthesizer, etc. The ICBR also has a core facility for isolation and production of monoclonal antibodies and also polyclonal antibodies.

Essential for this project is a large constant-temperature, fluorescent-lighted, sealed environmental chamber for culturing GS and GOGAT Arabidopsis mutant plants in a controlled atmosphere of 1% CO_2-air. We have successfully cultured Arabidopsis plants to maturity from seed in 4 to 6 weeks in this chamber. In addition, we have constructed 10 fluorescent-light shelves (3′ x 5′) for culturing wild-type or transgenic Arabidopsis plants in a constant temperature (22°C) culture room in normal air. All culturing of transgenic plants will be in the environmental chamber or culture room. No transgenic plants will be cultured outside of Dr. Schmidt's laboratory.

The culture room also has facilities for growing plant tissue cultures and also mass cultures of algae and bacteria, and it houses a Sharples continuous-flow centrifuge for harvesting large culture volumes. In addition, his laboratory has its own walk-in coldroom laboratory (104 sq. ft.), and a darkroom (55 sq. ft.) for development of autoradiograms and for viewing nucleic acids in gels with a transilluminator.

To facilitate the direction/advisement of his graduate students, Dr. Schmidt's office opens directly into his main laboratory where students have their laboratory benches and desks.